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DIZERTAČNÍ PRÁCE

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VÝZNAM CHROMOZOMÁLNÍCH ABERACÍ PRO HODNOCENÍ GENETICKÉHO RIZIKA EXPOZICE KARCINOGENŮM

ROLE OF CHROMOSOMAL ABERRATIONS TO EVALUATE GENETIC RISK OF EXPOSURE TO CARCINOGENS

Dizertační práce

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Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze,

Podpis:

Poděkování

Na tomto místě bych chtěla poděkovat všem, kteří mě v průběhu mé práce a studia podporovali a povzbuzovali, abych šla dál po započaté cestě.

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Abstrakt

Znečištěné ovzduší představuje celosvětově vážný problém spojený s riziky zejména nádorových onemocnění. Hodnocení negativních vlivů polycyklických aromatických uhlovodíků (k-PAU), reprezentovaných především benzo[a]pyrenem (B[a]P), na zdravotní stav obyvatelstva je analyzováno s využitím specifických biomarkerů, mezi něž patří i cytogenetické biomarkery časného účinku.

Tato práce shrnuje výsledky cytogenetických analýz, prováděných s využitím fluorescenční in situ hybridizace (FISH) (celochromozomové barvení chromozomů #1 a #4) a automatické obrazové analýzy mikrojader (MN). V průběhu studií bylo provedeno celkem 1304 analýz metodou FISH a 885 automatických obrazových analýz MN. Modelové skupiny, kterými byli městští strážníci, řidiči autobusů, pracovníci garáží, administrativní pracovníci, matky, novorozenci, zdravé děti a děti s astma bronchiale a pracovníci laboratoří, pocházely především z Prahy, ale i z Ostravy a Českých Budějovic. Studované lokality se významně lišily v koncentracích studovaných polutantů a celkovým typem znečištění. Expozice účastníků studií byla hodnocena s využitím personálního a stacionárního monitoringu. Ve studiích byl rovněž hodnocen vliv dalších faktorů, jako je věk, kouření či příjem vitaminů.

Výsledky získané metodou FISH ukázaly v Praze na vliv sezonních výkyvů koncentrací k-PAU na frekvenci chromozomálních aberací, stejně tak jako na významný negativní vliv koncentrací prachových částic o aerodynamickém průměru < 2,5 μm (PM2,5). Dílčí výsledky upozornily na změny v poměrech zastoupení stabilních a nestabilních chromozomálních aberací s věkem a riziko vyššího věku matek na zvýšené zastoupení stabilních aberací u zdravých novorozenců. V laboratoři byla zavedena automatická obrazová analýza MN, která se ukázala jako vhodná pomůcka pro aplikaci v biomonitorovacích studiích při hodnocení vlivu nízkých koncentrací k-PAU. Získané výsledky ve vztahu k dalším faktorům, jako je věk, pohlaví a kouření, odpovídaly mezinárodním výsledkům z manuálního hodnocení MN. Nově bylo započato hodnocení vlivu vysokých koncentrací k-PAU, kde se ukazuje, že míra poškození genetického materiálu souvisí s konkrétní hladinou genové exprese jednotlivých genů v organismu. Dílčí výsledky práce posloužily WHO pro stanovení rizikové hladiny B[a]P, která může indukovat poškození DNA.

Abstract (in English)

Air pollution is a serious worldwide problem associated with the risk of cancer. The negative effect of carcinogenic polycyclic aromatic hydrocarbons (c-PAHs), including benzo[a]pyrene (B[a]P), on human health is analyzed using specific biomarkers. Among them biomarkers of early effect play an important role.

This work summarizes the results of cytogenetic analyses performed by fluorescence in situ hybridization (FISH) (whole chromosome painting of chromosomes #1 and #4) and automated image analysis of micronuclei (MN). During the analyses a total set of 1304 samples was analyzed by the FISH method and 885 samples by the automated image analysis of MN. Studied groups including city policemen, garage men, bus drivers, administrative workers, mothers, newborns, healthy children and children with bronchial asthma and laboratory workers were from Prague, Ostrava and Ceske Budejovice. The locations significantly differed in levels of air pollutants and the type of air pollution. The exposure of participants of the study was assessed by personal and stationary monitoring. The impact of other factors including age, smoking or intake of vitamins was also evaluated in these studies.

The results obtained by the FISH method in Prague showed the impact of seasonal variability of concentrations of c-PAHs on the frequency of chromosomal aberrations as well as a negative impact of particulate matter of aerodynamic diameter < 2.5 μm (PM2.5). The results demonstrated that the ratio of stable and unstable chromosomal aberrations differed between newborns and mothers and that higher mother's age had a negative impact on the level of stable aberrations in newborns. The automated image analysis was set up in the laboratory as a sensitive method for assessment of the impact of low concentrations of c-PAHs. The frequency of MN in relation to age, gender and smoking was in agreement with international data obtained by visual scoring. The impact of high concentrations of c-PAHs on genetic damage suggested the important role of the level of expression of individual genes in the organism. The results were used by the WHO to specify the level of B[a]P that possesses the risk of induction of DNA damage.

Obsah

Příloha 3:

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1. Úvod

1.1. Cytogenetické biomarkery v molekulární epidemiologii

Molekulární epidemiologie je obor lékařských věd, který přispívá ke zlepšení znalostí o hlavních příčinách a mechanismech rozvoje onemocnění v populaci a zároveň připravuje preventivní strategie s cílem zlepšení zdravotního stavu obyvatelstva. Termín „molekulární epidemiologie“ byl poprvé použit ve spojení s molekulární epidemiologií chřipky, a to téměř před čtyřiceti lety (Kilbourne, 1973). V současnosti se molekulárně-epidemiologické studie zabývají například vztahy mezi kouřením a nádory plic, chronickou hepatitidou B a nádory jater či expozicí karcinogenním polycyklickým aromatickým uhlovodíkům (k-PAU) a rizikem rozvoje specifického nádorového onemocnění (Perera a kol., 1990). V mnoha studiích lze jasně definovat a kvantifikovat zdroj expozice, nicméně například studie zaměřené na vliv působení k-PAU jsou z tohoto hlediska značně komplikované. Výsledná expozice jedince je multifaktoriální a v souhrnu může být ovlivněna znečištěním ovzduší jak z dopravy, tak z lokálních topenišť a průmyslu, ale i stravou, či specifickým zaměstnáním (Wild a kol., 2008). Sumární expozice tedy závisí na lokalitě, kde studovaná populace žije, či pracuje, ale rovněž na životním stylu a rovněž na stravovacích návycích. Vyhodnocování vlivů znečištěného ovzduší je dále komplikováno faktem, že se jedná o bohatou směs celé řady látek, obsahující kromě k-PAU, reprezentovaných především benzo[a]pyrenem (B[a]P), a prachových částic různých frakcí i řadu dalších sloučenin, např. volatilní organické látky (VOC).

Pro hodnocení rizika expozice k-PAU se využívá velmi komplexní systém biomarkerů, který je v souvislosti s vývojem metodických přístupů stále doplňován (Binkova a kol., 1996; Smolders a kol., 2010). Biomarkery zahrnují všechny měřitelné odpovědi a interakce mezi biologickým systémem a faktory zevního prostředí. V zásadě je lze rozdělit do tří kategorií: biomarkery expozice, účinku a vnímavosti (Šrám a kol., 2007).

Mezi expoziční biomarkery řadíme látky a jejich metabolity, nebo produkty interakce xenobiotik a cílových buněk, popř. makromolekul, které lze detekovat v organismu. Do této kategorie patří například stanovení DNA aduktů, které bylo doporučeno pro hodnocení expozice B[a]P, a to i nízkým dávkám v ovzduší (Castano-Vinyals a kol., 2004; Topinka a kol.,

2007). Hlavní pomůcku při zjišťování konkrétních hodnot expozice k-PAU a VOC z ovzduší představuje personální a stacionární monitoring (Binkova a kol., 1998; Pinto a kol., 1998; Švecová a Šrám, 2007). Celosvětově je největší pozornost věnována B[a]P a benzenu, které představují hlavní rizikové faktory zmíněných k-PAU a VOC. Podle IARC (International Agency for Research on Cancer) jsou zároveň obě látky řazeny do první skupiny, tedy mezi látky karcinogenní pro člověka (IARC, http://monographs.iarc.fr).

Další kategorie zahrnuje biomarkery vnímavosti, které vypovídají o genetické predispozici jednotlivce či sledované skupiny k reakci na xenobiotika. Výsledkem je pak možnost různé odpovědi jedinců na stejnou expozici. Data o individuální vnímavosti jsou získávána prostřednictvím analýzy genetických polymorfismů především v genech, jejichž produkty mají vztah k metabolické aktivaci, či detoxifikaci xenobiotik. Příkladem těchto enzymů jsou epoxidhydroláza (EPHX1), hrající významnou roli v metabolické aktivaci, a glutation S-transferázy (GSTM1, GSTT1), uplatňující se v procesu detoxifikace. Polymorfismy v těchto genech byly studovány u řady exponovaných a kontrolních skupin (Novotna a kol., 2007; Sram a kol., 2007a; Rubes a kol., 2010). Z publikovaných výsledků vyplývá, že hrají významnou roli například i v modulaci poškození chromozomů (Iarmarcovai a kol., 2008).

Mezi další intenzivně studovanou kategorii patří biomarkery oxidačního poškození biomolekul, které je důsledkem oxidačního stresu vyvolaného působením reaktivních forem kyslíku (ROS). Oxidační stres je vyvolán nerovnováhou mezi hladinami antioxidantů a oxidantů v organismu. Mezi běžné antioxidanty, tedy látky snižující oxidační poškození, patří např. enzymy jako superoxiddismutáza (SOD), glutationperoxidáza, kataláza, či vitaminy A, C nebo E. Skupina oxidantů, které se naopak podílejí na vzniku ROS, zahrnuje jak endogenní, tak exogenní vlivy. Mezi exogenními faktory pak hrají nejvýznamnější roli vlivy životosprávy, znečištění ovzduší či infekce. Poškození v organismu, které může probíhat na úrovni nukleových kyselin, proteinů a rovněž i lipidů, je spojováno se vznikem nádorových onemocnění i nemocí dýchacího a kardiovaskulárního systému (Klauning a kol., 2010). V samotné DNA může dojít k modifikaci bází, z nichž nejčastější je 8-oxo-7,8-dihydro-2‘-deoxyguanosin (8-oxodG), který, pokud není včas rozpoznán a odstraněn, může být příčinou chybného párování. Rovněž může dojít k narušení cukr-fosfátové kostry a následnému zlomu (Cooke a kol., 2003). Vlivem působení ROS na proteiny vznikají karbonylové skupiny

především na postranních řetězcích aminokyselin prolinu, argininu, lysinu a treoninu. Následkem těchto modifikací může být například ztráta katalytické funkce enzymů vlivem zlomů či narušeného skládání proteinů (Dalle-Donne a kol., 2003). Dalším cílem ROS jsou lipidy, které jsou po primární oxidaci opakovaně peroxidovány, dokud nedojde k ukončení tohoto děje antioxidantem (Montuschi a kol., 2004). Hlavním cílem pro ROS jsou membránové lipidy, jejichž peroxidací dochází ke změně vlastností buněčných membrán. Působením ROS na arachidonovou kyselinu dochází k odštěpování isoprostanů, které jsou zároveň hlavním markerem peroxidace lipidů, konkrétně pak především 15-F2t-isoprostan (15-F2t-IsoP) (Roberts a Morrow, 2000).

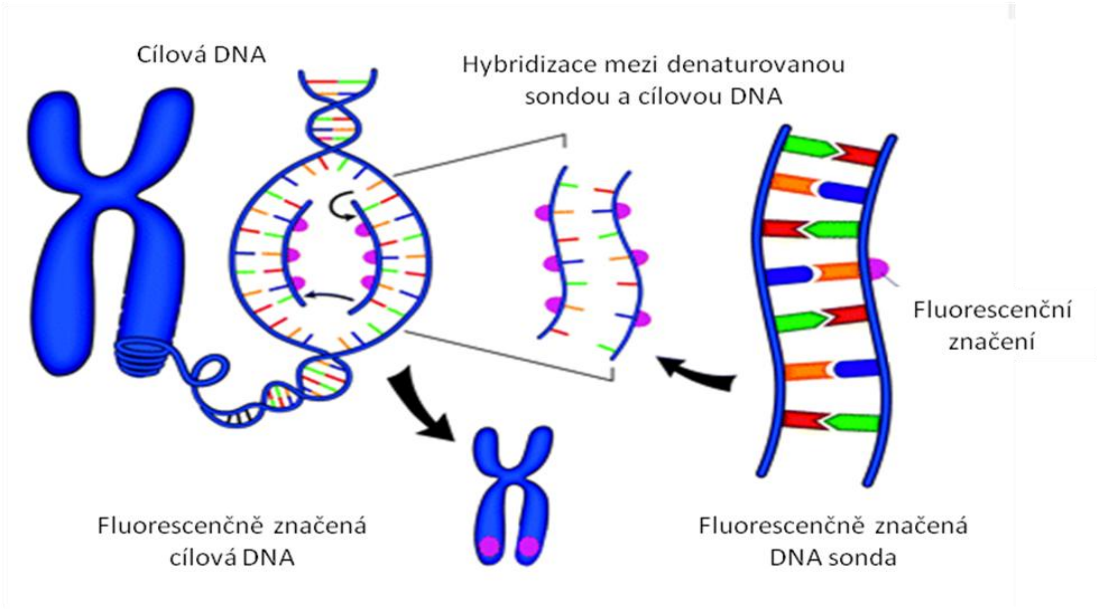
V neposlední řadě se dostáváme k biomarkerům účinku, konkrétně tzv. biomarkerům časného účinku, které umožňují vyhodnotit zvýšené riziko vzniku zejména nádorových onemocnění až desetiletí před jejich rozvojem. Mezi biomarkery účinku řadíme kromě mutací v konkrétních genech, jako jsou *HPRT*, *p53* a *p21*, i výsledky metodik zaměřených na komplexnější screening chromozomálních přestaveb a poškození v lidských periferních lymfocytech (PBL). V průběhu let byly využívány metodiky zaměřené na výměnu sesterských chromatid (SCE), konvenční cytogenetická analýza (CCA), fluorescenční in situ hybridizace (FISH) a analýza mikrojader (MN) jak v klasickém, tak nově v automatickém pojetí. Asi nejpoužívanější metodikou v naší republice byla od konce 70. let využívaná CCA, zaměřená především na studium nestabilních chromozomálních aberací reprezentovaných chromozomovými a chromatidovými zlomy. Metodika byla využívána zejména k hodnocení vlivu genotoxických látek u profesionálně exponovaných osob (Rossner a kol., 1995; Sram a kol., 2004a). Získané výsledky rovněž ukázaly na její vhodnost k hodnocení rizika vzniku nádorových onemocnění (Bonassi a kol., 2008) a při dlouhodobém systematickém studiu též k hodnocení vlivů měnících se environmentálních expozic a faktorů životního stylu, jak bylo ukázáno u skupiny 3402 dětí analyzovaných v letech 1984-1999 (Rossner a kol., 2002). Pro hodnocení menších souborů se však později ukázala vhodnější aplikace metodiky FISH (Sram a kol., 2007b), která je podrobněji předmětem následující kapitoly. Rovněž analýza MN, která představuje alternativní metodiku k analýze metafází při CCA, je předmětem podstatné části tohoto literárního úvodu.

Na závěr kapitoly jen připomenu důležitost sledování již zmíněných faktorů životního stylu, jejichž ukazateli jsou především příjem vitaminů, hladiny lipidů a kotininu. Mezi další studované faktory patří i body mass index (BMI), úroveň vzdělání či věk účastníků studií. Pro úplnou komplexnost celého procesu mutageneze a karcinogeneze je nutné si ještě uvědomit, že na pozadí všech procesů vedoucích k poškození DNA probíhají velmi účinné reparační procesy, které mohou být dalším studovaným cílem. Velký význam se v posledních letech přikládá rovněž studiu genové exprese, jejíž míra vyplývá z konkrétních potřeb a podmínek, ve kterých se organismus nachází.

1.2. Fluorescenční in situ hybridizace (FISH), její priority a aplikace v biomonitorovacích studiích

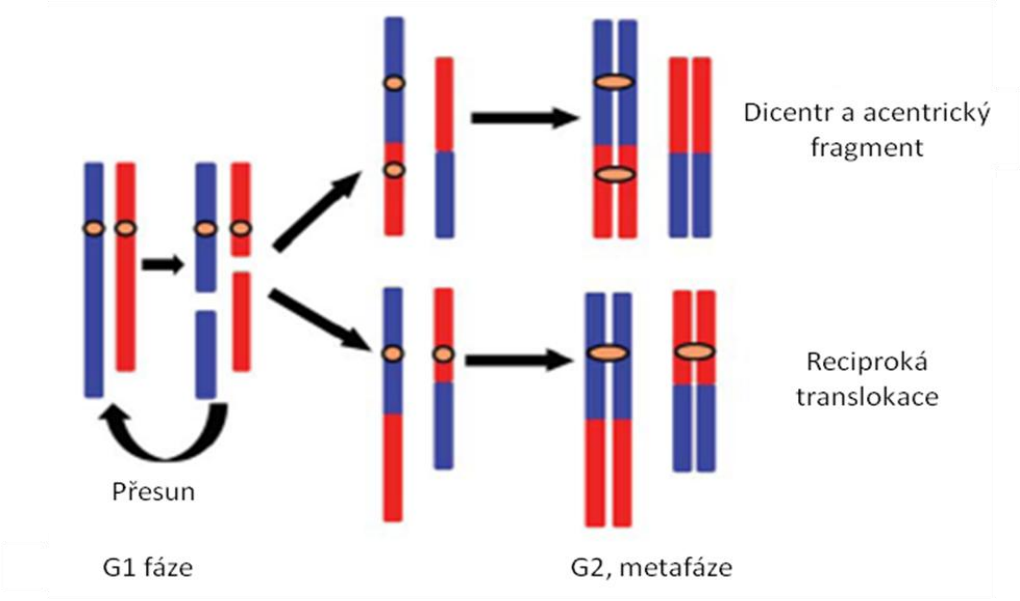
V porovnání s CCA je FISH relativně novou metodikou, která byla popsána v roce 1986 jako metoda pro detekci a klasifikaci chromozomálních aberací (Pinkel a kol., 1986). Princip metodiky, založený na vzájemné hybridizaci denaturované fluorescenčně značené DNA sondy s denaturovanou cílovou DNA, je znázorněn na obrázku 1. Obecně se dnes jedná o analýzu s velmi širokým uplatněním, které je dáno jak použitím různých typů fluorescenčních sond, tak možnou aplikací na metafázní, i na interfázní chromozomy. Vedle bohatého použití v klinické a nádorové cytogenetice našla metodika uplatnění rovněž v molekulární epidemiologii. Její výhoda, kterou obohatila spektrum již užívaných biomarkerů, spočívá v možnosti detekovat rutinně stabilní chromozomální aberace, zejména translokace, které bylo možno dříve stanovovat jen velmi omezeně. Chromozomální translokace vznikají jako následek dvouřetězcových zlomů, které byly chybně opraveny. V nejjednodušším případě mohou jako následek těchto zlomů na různých chromozomech vzniknout celkem 3 druhy přestaveb, z nichž nejpravděpodobnější je reciproká translokace (Tucker, 2010) (obrázek 2).

V biomonitorovacích studiích se dnes aplikuje především celochromozomové barvení největších chromozomů tak, aby procento genomu, které bylo obarveno, bylo co možná nejvyšší. Malé chromozomy jsou v tomto ohledu nevýhodné, neboť je pak nutné hodnotit vyšší počty metafází. Testování různých dvojic barvených chromozomů u PBL exponovaných 0.3 a 1 Gy gama záření, do kterého byly zahrnuty chromozomy 1-7, 11, 13, 16 a 18, ukázalo,



Obr.1:

Princip metodiky FISH, založený na vzájemné hybridizaci denaturované fluorescenčně značené DNA sondy s denaturovanou cílovou DNA.



Obr.2:

Chromozomální zlomy ve dvou nehomologních chromozomech mohou vést při chybné reparaci ke vzniku dicentrického chromozomu a acentrického fragmentu, nebo reciproké translokaci (Tucker, 2010).

že pro hodnocení lze použít libovolnou kombinaci chromozomů (Garcia-Sagredo a kol., 1996), nicméně další práce toto tvrzení znovu diskutují jak ve vztahu k vyšším dávkám záření (Boei a kol., 1997), tak ve vztahu k různým klastogenům, a zejména k-PAU (Beskid a kol., 2006a; Bocskay a kol., 2007; Orjuela a kol., 2010). Klíčové pro aplikace celochromozomového barvení v biomonitorovacích studiích v různých laboratořích je správná klasifikace a vyhodnocení chromozomálních aberací s využitím PAINT protokolu (Protocol for Aberration Identification and Nomenclature) (Tucker a kol., 1995) a rovněž výpočet genomické frekvence translokací, která umožňuje mezilaboratorní porovnání výsledků i při barvení různých chromozomů (Lucas a Sachs, 1993). I přes nesporné výhody při hodnocení, které umožňuje v porovnání s CCA poměrně jednoduše hodnotit velké počty metafází, zůstává zatím hlavním handicapem kromě ceny i možnost plné automatizace. První fázi vyhledávání metafází se již dříve podařilo automatizovat, nicméně vyhodnocování konkrétních typů přestaveb bylo zatím úspěšné jen v souvislosti s dicentrickými chromozomy (Schunck a kol., 2004). Hodnocení širokého spektra chromozomálních přestaveb je stále závislé na lidském faktoru (Tucker, 2010).

Metodika FISH našla své uplatnění v radiační biologii (Tucker, 2001), studiích hodnotících vliv expozice nejrůznějších chemických látek u profesionálně exponovaných osob a v neposlední řadě při hodnocení vlivu environmentálních expozic, především vlivu k-PAU. V radiobiologických, ale i dalších studiích na lidské populaci je využíváno zejména toho, že translokace jsou při hodnocení s časovým odstupem výhodnější vzhledem k jejich stabilitě v porovnání s dicentrickými chromozomy, jejichž frekvence výrazně klesá v čase, ale i s každým dalším dělícím cyklem v průběhu kultivace (Edwards a kol., 2005). Hodnocení translokací bylo využito například i při pozdějším hodnocení osob exponovaných při havárii elektrárny v Černobylu (Edwards a kol., 2004). V jiných studiích byly studovány zejména vlivy expozice pesticidům (Tucker a kol., 2003; Zeljezic a kol., 2009), kovům jako je olovo, kadmium či chrom (Palus a kol., 2003; Maeng a kol., 2004), ionizačního záření u pilotů (Yong a kol., 2009), či vliv expozice styrenu nebo perchlorethylenu (Naccarati a kol., 2003; Tucker a kol., 2011). Vliv znečištěného ovzduší, konkrétné k-PAU na stabilní chromozomální aberace byl několikrát studován v New Yorku u skupiny afroamerických a dominikánských novorozenců (Bocskay a kol., 2005; Bocskay a kol., 2007; Orjuela a kol., 2010). Rovněž další

studie přinášejí výsledky frekvence translokací u novorozenců (Pluth a kol., 2000; Sigurdson a kol., 2008).

V naší republice byla metodika s využitím celochromozomového barvení chromozomů 1 a 4, které se staly zároveň nejčastěji barvenými chromozomy (Sigurdson a kol., 2008), poprvé použita k hodnocení translokací u zdravotnického personálu (Rubes a kol., 1998). Výsledky ukázaly na významné signifikantní rozdíly v genomické frekvenci translokací na sto hodnocených buněk (F_G/100) při porovnání zdravotnického personálu a kontrol a zároveň na vyšší citlivost metodiky v porovnání s CCA.

Další práce pak hodnotí vliv nízkých dávek ionizujícího záření na chromozomální aberace u pracovníků jaderných elektráren v naší republice (Sram a kol., 2006). S použitím CCA a FISH využívající celochromozomové a centromerické sondy bylo potvrzeno, že nízké dávky ionizujícího záření nepředstavují rizikový faktor pro poškození genetického materiálu.

Hodnocení vlivu klastogenního účinku nejrůznějších chemických látek u profesionálně exponovaných osob bylo u nás již dříve systematicky studováno s využitím CCA (Rossner a kol., 1995; Sram a kol., 2004a). Cytogenetická analýza využívající rovněž celochromozomové barvení u osob exponovaných 1,3-butadienu, akrylonitrilu, ethylbenzenu, benzenu a k-PAU z ovzduší ukázala na vyšší citlivost metodiky a zároveň vhodnost pro další využití při hodnocení vlivů znečištěného ovzduší (Sram a kol., 2004b; Beskid a kol., 2006a), na které se pak další práce zaměřují (Beskid a kol., 2007; Sram a kol., 2007a; Sram a kol., 2007b). Zmíněné práce prováděné především na modelových skupinách městských strážníků potvrzují škodlivý vlivy k-PAU na genetický materiál. Rovněž se zdá, že významnou roli hrají sezonní rozdíly v koncentracích k-PAU.

Kromě vlivu studovaných látek v biomonitorovacích studiích umožnily nashromážděné výsledky vyhodnotit vliv dalších faktorů na frekvenci translokací. Mezi nejvýznamnější patří věk, který byl průběžně studován na vzorcích kontrolní populace (Sorokine-Durm a kol., 2000; Whitehouse a kol., 2005). Základní frekvence translokací a zároveň vliv věku, pohlaví, rasy a kouření byly hodnoceny v mezinárodním souboru 1933 osob z 16 laboratoří světa, které shrnují výsledky napříč celým věkovým spektrem (Sigurdson a kol., 2008).

Vedle profesionálně exponovaných osob, městských strážníků a kontrolních skupin byla provedena pilotní studie hodnotící vliv znečištění ovzduší u matek a jejich dětí v Teplicích a Prachaticích, které představují modely znečištěné průmyslové a čisté zemědělské oblasti (Pedersen a kol., 2006). Z cytogenetických metodik zde byla provedena FISH a rovněž manuální hodnocení MN s využitím nefluorescenčního barvení, které ukázalo na signifikantní rozdíly mezi lokalitami.

1.3. Analýza mikrojader (MN) a její metodické varianty

Analýza mikrojader je dnes pravděpodobně nejčastěji užívanou metodikou sloužící k hodnocení frekvence chromozomálních aberací u různě exponovaných lidských populačních skupin, ale i k vyhodnocení cytotoxicity či genotoxicity studovaných látek na buněčných liniích. Stanovení frekvence mikrojader dnes představuje především metodiku k hodnocení chromozomálních zlomů, které jsou vyjádřeny jako acentrické fragmenty uvnitř mikrojádra, ale i k hodnocení chromozomálních ztrát.

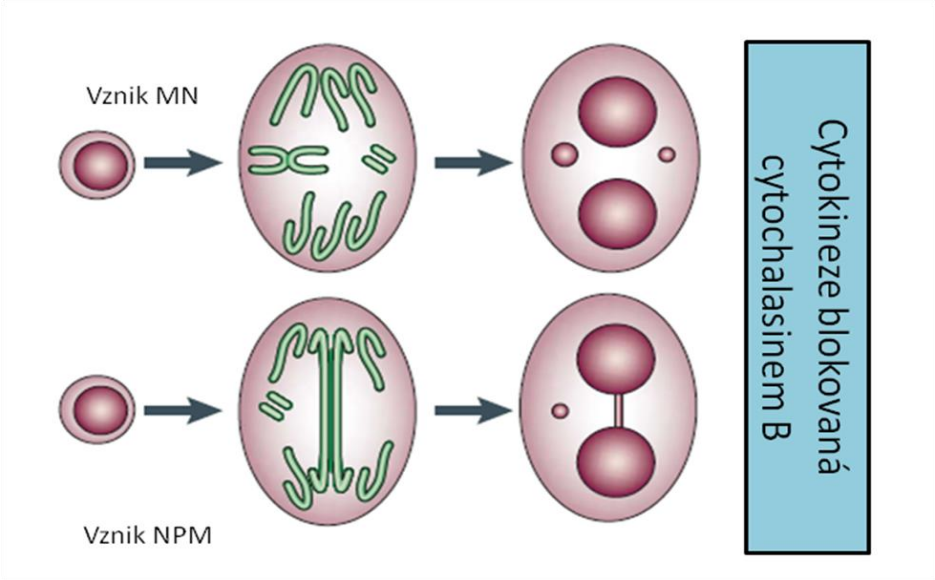
Kořeny této metodiky sahají až do roku 1959, kdy byla použita pro stanovení chromozomálních aberací u Vicia faba (Evans a kol., 1959). Další významnou událostí pro budoucí širokou aplikaci bylo využití metodiky pro stanovení chromozomálních aberací v kultivovaných PBL (Countryman a Heddle, 1976). Během dalších let byla metodika použita v celé řadě aplikací i obměnách: in vivo i in vitro, s využitím různých způsobů barvení buněk a vedle PBL byly využívány též epiteliální buňky, erytrocyty či fibroblasty. Pro dnešní podobu metodiky měla zásadní význam aplikace cytochalasinu B (Fenech a Morley, 1985), který blokuje vznik mikrofilamentárního kontraktilního prstence při dělení buněk a výsledkem jsou pak dvoujaderné buňky (DB), které jsou optimální pro hodnocení mikrojader. Působení cytochalasinu B na cytokinesi v dalších buněčných cyklech vede ke vzniku vícejaderných buněk. Zastoupení jednojaderných až čtyřjaderných buněk má význam zejména pro kontrolu proliferace stanovením jaderného dělícího indexu.

Přestože hlavním cílem většiny aplikací mikronukleového testu je skórování již zmíněných mikrojader, byla metodika v průběhu dalších let podrobně rozpracována tak, aby nabídla co nejširší využití (Fenech, 2000; Fenech, 2006; Fenech, 2007) (obrázky 3 a 4). Metodika může

být v současné době využita k hodnocení chromozomálních zlomů, nehomologní reparace, ztrát chromozomů, genové amplifikaci, ale i pro stanovení frekvence nekrotických a apoptotických buněk. Metodika je rovněž použitelná pro stanovení tzv. nukleoplasmatických můstků (NPM), které indikují přítomnost dicentrických chromozomů vzniklých fúzí dvou chromozomů, které po poškození nejsou ukončeny telomerou. Přítomnost NPM, které jsou často provázeny přítomností mikrojádra, je možné pozorovat výhradně v dvoujaderných buňkách a nikoliv v jednojaderných, které jsou rovněž v některých studiích hodnoceny.

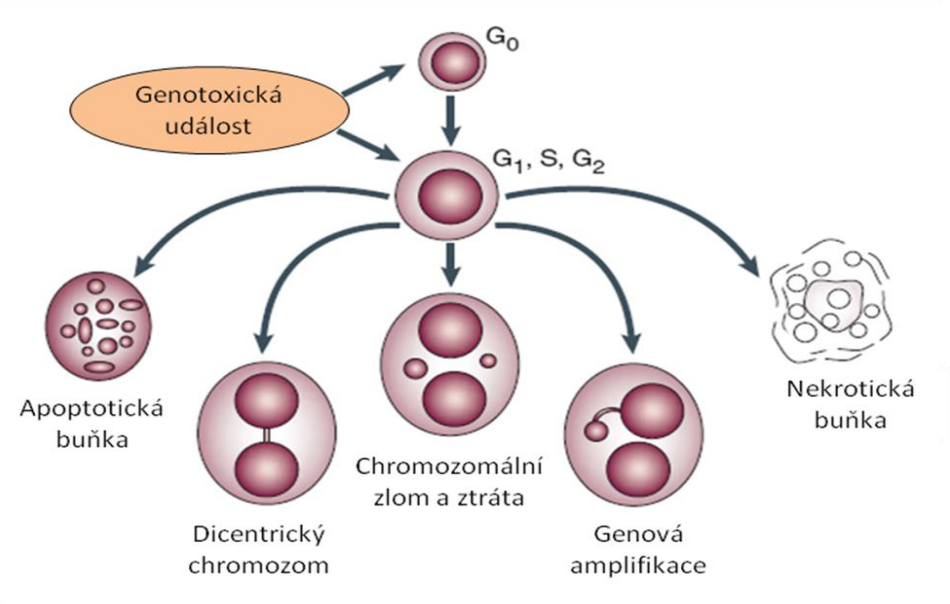
Všechny zatím zmíněné typy buněk mohou být dobře pozorovatelné v nefluorescenčně barvených preparátech. Propojení s metodou FISH však nabídlo další možnosti. Aplikace centromerických, či telomerických sond může přinést konkrétnější informaci o obsahu mikrojádra (obrázek 5), či přítomnosti konkrétního chromozomu. Takto bylo například identifikováno, že cca 62% MN obsahuje telomery a reprezentuje tedy acentrické fragmenty a u cca 22% MN byly identifikovány jak telomery, tak i centromera. Zbylá MN bez fluorescenčního signálu reprezentují intersticiální DNA fragmenty (Lindberg a kol., 2007). Další identifikací konkrétních chromozomů ukázalo proti odhadům vyšší frekvenci gonozomů v MN (Catalan a kol., 2006).

Analýza mikrojader představuje relativně jednoduchou, nicméně časově náročnou metodiku. Ve většině studií je hodnoceno 1000 DB, ale pro zvýšení přesnosti výsledku nalezneme i práce, kde bylo hodnoceno i dva či více tisíc DB. Zejména z těchto důvodů, ale i z důvodu rizika subjektivní interpretace kritérií pro hodnocení, se začaly objevovat snahy o automatizaci tohoto hodnocení. První automatická analýza s využitím průtokového cytometru byla publikována již v roce 1982 (Hutter a Stohr, 1982). V současnosti je tento druh analýzy užíván jak pro hodnocení MN v erytrocytech, tak i v savčích buněčných kulturách (Dertinger a kol., 2011; Avlasevich a kol., 2011). Jiná cesta k automatizaci metodiky se od roku 1990 zaměřila na obrazovou analýzu (Tates a kol., 1990). V několika dalších studiích pak byl diskutován především problém s účinností vyhledávání DB, vyhledávání drobných MN a identifikace MN v těsné blízkosti jádra (Castelain a kol. 1993; Verhaegen a kol., 1994; Bocker a kol., 1995; Bocker a kol., 1996). Rozvoj počítačových technologií nabídl pokročilejší automatické systémy. V současné době zaznamenaly největší rozvoj systémy Metafer MNScore (MetaSystems) (Schunck a kol., 2004; Varga a kol., 2004; Rossnerova a



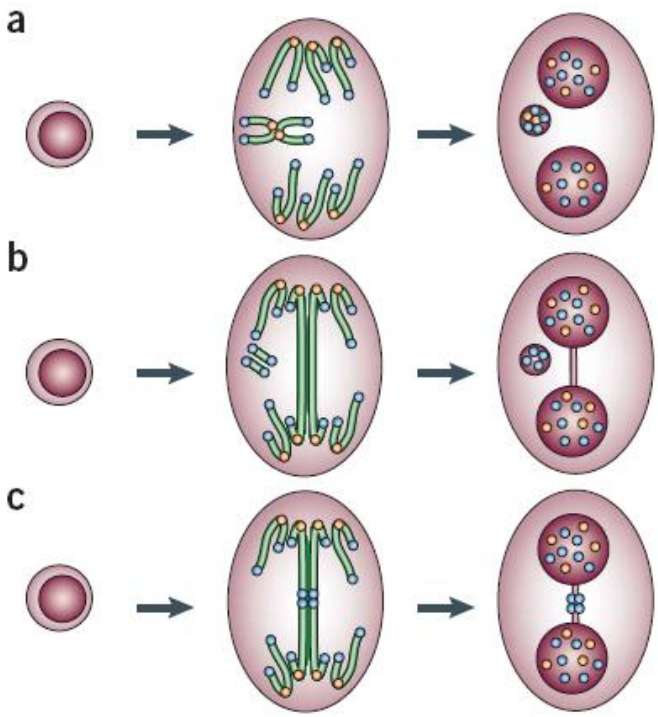
Obr.3:

Vznik mikrojader a nukleoplasmatických můstků v buňkách s cytokinezí blokovanou cytochalasinem B (Fenech, 2007).



Obr.4:

Možné varianty kultivovaných buněk s blokovanou cytokinezí po cytotoxickém či genotoxickém působení chemických látek: buňka s probíhající apoptozou, NPM vyjadřující dicentrický chromozom, buňka s chromozomalním zlomem, či ztrátou, genová amplifikace a nekrotická buňka (Fenech, 2007).



Obr.5:

Aplikace centromerické a telomerické sondy na DB: a) důkaz ztráty chromozomu, b) identifikace chromozomového zlomu a NPM, c) NPM vzniklý fúzí telomer dvou chromozomů (Fenech, 2007).

kol., 2011a) (obrázek 6) a PathFinder^{TM}Cellscan^{TM} (IMSTAR) (Decordier a kol., 2009; Decordier a kol., 2011), které se zásadně liší především barvící technikou, při které nejúčinněji pracují. První zmíněný systém využívá především fluorescenčně 4´-6-diamidino-2-fenylindolem (DAPI) barvených preparátů, zatímco druhý scanuje a hodnotí vzorky barvené tradičně Giemsou. Podrobnosti o prvním systému jsou zároveň předmětem této dizertační práce (viz příloha 7).

Pro úplnost tohoto metodického přehledu je třeba však ještě zmínit rozvíjející se laserovou skenovací cytometrii, která kombinuje průtokovou cytometrii s obrazovou analýzou (Smolewski a kol., 2001; Darzynkiewicz a kol., 2011).

Obr.6:

Automatický skenovací systém Metafer s přídavným zařízením umožňující kontinuální skenování až 80 preparátů (Rossnerova a kol., 2011a).

1.4. Hlavní směry aplikace mikronukleového testu

Aplikace MN testu je velmi široká, což částečně vyplynulo již ze zmíněných metodických variant. Zadáním klíčového slova „micronucleus“ do internetové database PubMed získáme dnes více než 7000 výsledků in vitro či in vivo prací provedených s nejrůznějším biologickým materiálem na jedno- či dvoujaderných buňkách za účelem testování genotoxických účinků širokého spektra látek. Následující zkácený přehled se vzhledem k zaměření této práce bude týkat především lidských populačních studií, kde byla hodnocena MN v DB PBL.

Mezi zmíněným druhem studií zaujímají přední místo ty, které vznikly v rámci projektu HUMN započatého v roce 1997 (Human MicroNucleus project, event. The International Collaborative Project on Micronucleus Frequency in Human Populations - http://ehs.sph.berkeley.edu/holland/humn/). Jeho hlavními cíli se stalo shromažďování dat z různých laboratoří za účelem porovnat a optimalizovat metodické protokoly, stanovit hlavní proměnné, které ovlivňují frekvence MN, a vyhodnotit rizika vzniku nejrůznějších onemocnění (Fenech a kol., 1999). K hlavím úspěchům projektu HUMN, který pracoval

s databází více než 7000 osob, patří především stanovení základních frekvencí MN v neexponované populaci a vyhodnocení vlivu věku a pohlaví (Bonassi a kol., 2001), srovnávací studie hodnocení preparátů připravených ze stejné kultury provedená ve 34 laboratořích světa (Fenech a kol., 2003a), detailní popis kriterií pro hodnocení (Fenech a kol., 2003b), vyhodnocení vlivu kouření na frekvenci MN (Bonassi a kol., 2003) a důkaz, že zvýšená frekvence MN je prediktivním biomarkerem nádorových onemocnění (Bonassi a kol., 2007).

Na projekt HUMN navazuje od roku 2007 další projekt označený jako $HUMN_{XL}$ (The International Collaborative Project on the Micronucleus Frequency in Human Exfoliated Buccal Cells), který se alternativně zaměřuje na hodnocení frekvence mikrojader v bukální sliznici ústní dutiny, jejíž odběr je na rozdíl od PBL neinvazivní. Primární cíle jsou podobné jako u HUMN projektu, přičemž metodické detaily byly již publikovány (Thomas a kol., 2009). Současná databáze zahrnuje více než 5000 osob (Fenech a kol., 2011a).

Kromě již zmíněného vlivu kouření na frekvenci MN, který byl prokázán pouze u silných kuřáků (kouřících 30 a více cigaret za den) (Bonassi a kol., 2003), je genotoxické poškození studováno i ve vztahu k dalším faktorům životního stylu (Fenech a Bonassi, 2011b). Nadměrná konzumace alkoholu je dlouhodobě spojována s genotoxickým efektem, jehož míra je dále ovlivněna aktivitou metabolických enzymů alkoholdehydrogenázy-2 (ADH1B), cytochromu P4502E1 (CYP2E1) a acetaldehyd dehydrogenázy (ALDH2) (Ishikawa a kol., 2006a; Ishikawa a kol., 2007). Studován je rovněž vliv extrémní fyzické zátěže (Stefanie a kol., 2008). Největší pozornost je však věnována vlivu výživy (Thomas a kol., 2011). Nedávná studie shrnula, že nízký příjem vápníku, folátů, niacinu, vitaminů A a E a β-karotenu a vysoký příjem kyseliny pantotenové, biotinu a riboflavinu jsou signifikantně asociovány s nárůstem genomové instability (Fenech a kol., 2005). V jiných studiích bylo prokázáno, že vegetariánství nemá klíčový vliv na výslednou frekvenci MN (Fenech a Rinaldi, 1995; Kazimirova a kol., 2006). Velká pozornost je v rámci evropského projektu NewGeneris věnována v poslední době vlivu výživy matek v průběhu těhotenství na zdravotní stav novorozenců (Merlo a kol. 2009).

Asociace mezi zvýšenou frekvencí MN a rizikem nádorových onemocnění byla rovněž zmíněna jako jeden z výsledků projektu HUMN (Bonassi a kol., 2007), což potvrzuje i další

práce (Bonassi a kol., 2011). Další studie však ukázaly, že poškození genetického materiálu je asociováno i s dalšími zdravotními problémy, jako jsou kardiovaskulární onemocnění, cukrovka či metabolický syndrom (Andreassi a kol., 2011). Zvýšená frekvence MN s jejich rozdílným původem byla rovněž asociována s neurodegenerativními onemocněními, jako je Alzheimerova či Parkinsonova choroba. U Alzheimerovy choroby byla zjištěna zvýšená frekvence aneuploidií, zatímco MN u Parkinsonovy choroby pocházejí převážně z chromozomálních zlomů (Migliore a kol., 2011). V poslední době se rovněž diskutuje o využití hodnocení MN v PBL a reprodukčních tkáních jako biomarkeru rizika infertility a komplikací v průběhu těhotenství (Fenech, 2011c).

Analýza MN v PBL je využívána v četných in vivo biodosimetrických studiích hodnotících vliv expozice ionizujícímu záření. Vedle analýzy dicentrických chromozomů je to alternativní metoda, jejíž hlavní výhodou je rychlejší hodnocení (Vral a kol., 2011). Alternativou je i kombinace s FISH, kdy jsou barveny centromery, čehož bylo využito např. při monitoringu pracovníků jaderných elektráren (Thierens a kol., 1999), nebo při monitoringu zdravotnického personálu exponovaného X- a γ-záření (Thierens a kol., 2000). Současný stav aplikace se zaměřil na využití automatické obrazové analýzy MN jako pomůcky k odhadu individuální expozice ionizujícímu záření při rozsáhlejším ozáření populace (Thierens a Vral, 2009; Willems a kol., 2010).

Velké množství prací se věnovalo vlivu nejrůznějších profesionálních expozic na genotoxické poškození. Nedávná studie se věnovala vlivu expozici styrenu na frekvenci MN (Hanova a kol., 2010). Jiná studie prokázala nepříznivý vliv expozici formaldehydu (Ladeira a kol., 2011). Velká pozornost byla věnována vlivu pesticidů, jejichž chemická variabilita se odráží i v heterogenitě získaných výsledků (Bolognesi a kol., 2011). Mezi dalšími studiemi zaujímají významné místo ty, které hodnotí vliv znečištěného ovzduší. Milosevic-Djordjevic vyhodnocuje pokles frekvence MN v populaci při porovnání novorozenců, kteří se narodili 12 měsíců po bombardování v Srbsku, které vedlo k vážné kontaminaci ovzduší, vody a půdy, s novorozenci narozenými v roce 2006 (Milosevic-Djordjevic a kol., 2007). Zvýšená frekvence poškození DNA byla zjištěna u žen z průmyslové oblasti v Číně v porovnání se ženami ze zemědělské oblasti (Ishikawa a kol., 2006b). Stejný trend měly výsledky pilotní studie u dětí a matek z Teplic a Prachatic v naší republice

(Pedersen a kol., 2006), na rozdíl od relativně vysokých frekvencí MN u novorozenců ze zemědělských oblastí v Mexiku v porovnání s městskými novorozenci (Levario-Carrillo a kol., 2005). Zajímavou alternativu k lidským studiím přinášejí studie hodnotící MN v silně znečištěných oblastech světa s využitím rostlinného materiálu (Prajapati a Tripathi, 2008; Mariani a kol. 2009).

Zvýšená frekvence MN byla konzistentně prokazována ve vztahu ke genetickým polymorfismům reparačních genů *ERCC2* a *XRCC1*, dále polymorfismu v genu *CYP2E1*, jehož produkt je spojován s metabolickou aktivací xenobiotik, a genu *MTR* v souvislosti s metabolismem folátů (Dhillon a kol., 2011). Předchozí review ve vztahu k formování mikrojader ukázalo, že polymorfismy v genech *EPHX1, GSTM1 a GSTT1* hrají rovněž významnou roli ve vztahu k jejich modulaci (Iarmarcovai a kol., 2008). Existuje obrovské množství prací, hodnotících vlivy genetických polymorfismů na tvorbu MN, nicméně většinou se jedná o malé soubory, které přinášejí někdy i protichůdné výsledky. Velký význam pro interpretaci mají především velké soubory jako například v práci Kirsch-Volders, v níž jsou analyzovány výsledky ze sedmi laboratoří ve vztahu ke glutation S-transferázovým genům *GSTM1* a *GSTT1* u dospělých jedinců (Kirsch-Volders a kol., 2006). Biomonitorovací studie věnující se genetickým polymorfismům u dětí ve vztahu k MN jsou zatím poměrně vzácné (Decordier a kol., 2007).

Mezi relativně nové směry zájmu ve vztahu k MN patří studie zaměřené na transkriptomiku. S pomocí speciálního softwaru MetaCore jsou vytvářeny sítě vzájemných vztahů mezi geny zúčastněnými při formování MN, což umožňuje studovat mechanistické vztahy (van Leeuwen a kol., 2011). Pravděpodobně první takto studovanou populaci, kde byla hodnocena úroveň transkripce, patří skupina dětí a dospělých z naší republiky s rozdílnou expozicí znečištěnému ovzduší (van Leeuwen a kol., 2006, van Leeuwen a kol., 2008).

Pro úplnost tohoto stručného přehledu je třeba ještě uvést, že velmi významnou aplikací mikronukleového testu je jeho využití v nesčetných in vitro studiích pro testování genotoxicity celé řady chemických látek, včetně B[a]P (Fowler a kol., 2010) či organických extraktů ze znečištěných oblastí (Roubicek a kol., 2007). Tyto testy, prováděné na nejrůznějších buněčných liniích, či PBL se řídí směrnicemi OECD (Organisation

for Economic Co-operation and Development) (OECD guideline 487, 2007). Zcela novou oblastí výzkumu se v poslední době stává testování genotoxicity nanomateriálů, které jsou definovány jako látky, jejichž jeden rozměr je menší než 100 nm. Pro tyto testy byly již vydány předběžné směrnice (OECD preliminary guideline, 2009) a výsledky prvních 21 studií jsou shrnuty v nedávno publikovaném review (Gonzalez a kol., 2011).

2. Cíle studie

Práce se především zabývá vlivem karcinogenních polycyklických aromatických uhlovodíků (k-PAU), zejména benzo[a]pyrenu (B[a]P), na míru poškození genetického materiálu u různě zatížených populačních skupin naší republiky. Kromě B[a]P jsou studovány vlivy dalších znečišťujících látek, jako jsou prachové částice o aerodynamickém průměru < 2,5 μm (PM2,5) či benzen. Faktory životního stylu reprezentované například příjmem vitaminů či hladinou kotininu a vliv věku jsou ve studiích rovněž analyzovány. K hodnocení bylo využito zavedené metody FISH a nově též automatické obrazové analýzy MN.

Hlavní cíle práce byly formulovány následovně:

1. u modelových populačních skupin analyzovat vliv nízkých koncentrací k-PAU na frekvence chromozomálních aberací;
2. vyhodnotit zastoupení jednotlivých druhů chromozomálních aberací identifikovaných metodou FISH v závislosti na věku;
3. zavést automatickou obrazovou analýzu MN pro využití v biomonitorovacích studiích;
4. vyhodnotit úroveň poškození genetického materiálu s využitím automatické obrazové analýzy MN v závislosti na hladině expozice studovaným znečišťujícím látkám;
5. zhodnotit faktory, které frekvenci chromozomálních aberací modifikují;
6. porovnat efektivnost obou aplikovaných metod pro další využití v biomonitorovacích studiích.

3. Použité metody

Znečištění ovzduší

Personální monitoring expozice znečištěnému ovzduší (Binkova a kol., 1998; Švecová a Šrám, 2007)

Stationární monitoring znečištění ovzduší (Pinto a kol., 1998)

HPLC kvantitativní chemická analýza k-PAU (EPA Report 1999; EN ISO CSN IEC 17025)

Cytogenetické metodiky

Kultivace lidských periferních lymfocytů (PBL) pro hodnocení chromozomů v metafázi (Sorsa a kol., 1994; Rossner a kol., 2002)

Kultivace PBL pro hodnocení dvoujaderných buněk (DB) (Fenech a Morley, 1985)

Konvenční cytogenetická analýza (Carrano a Natarajan, 1988; Rossner a kol., 2002)

Fluorescenční in situ hybridizace - celochromozomové barvení chromozomů #1 a #4 (Rubes a kol., 1998; Beskid a kol., 2007) (obrázek 7)

Analýza chromozomálních aberací FISH preparátů (Morton, 1991; Lucas a Sachs, 1993; Tucker a kol., 1995)

Automatická obrazová analýza mikrojader (Schunck a kol., 2004; Varga a kol., 2004; Rossnerova a kol., 2009a) (obrázek 8)

Ostatní metodiky

Isolace DNA ze slin (ORAgene DNA kit)

Analýza genetických polymofismů (TaqMan Real-Time PCR metody: TaqMan Gene Copy Number Assay [PN4331182] a TaqMan Drug Metabolism Genotyping Assay [C_14938_30 a C_11638783_30], Applied Biosystems)

Analýza genové exprese (isolace celkové RNA s využitím systému LeukoLOCK^{TM}, měření koncentrace pomocí Nanodrop ND-1000 spektrofotometru, kontrola integrity RNA bioanalyzerem Agilent 2100, reverzní transkripce – ROCHE kit, kvantitativní PCR (qPCR) s využitím 7900HT Fast Real-Time PCR systému)

Stanovení hladiny 8-oxodG v moči (Yin a kol., 1995; Rossner Jr. a kol., 2008a)

Stanovení hladiny karbonylových skupin v krevní plasmě (Buss a kol., 1997; Rossner Jr. a kol., 2007)

Stanovení hladiny 15-F2t-IsoP v krevní plasmě (Rossner Jr. a kol., 2009)

Stanovení DNA aduktů polyaromatických látek metodou ^{32}P – postlabelingu (Binkova a kol. 2007)

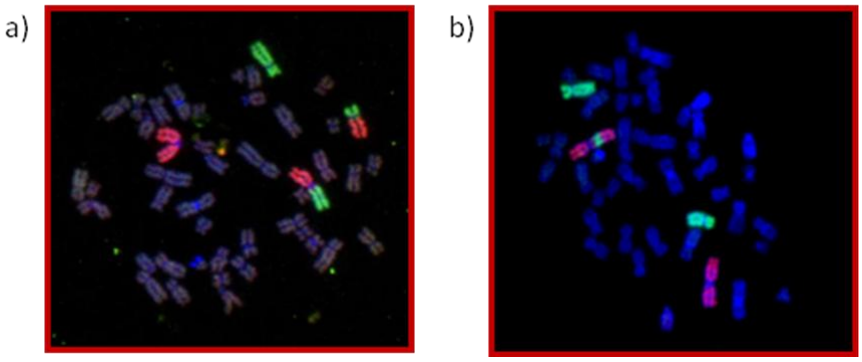
Radioimunochemické stanovení kotininu (Langone a van Vunakis, 1982)

Stanovení vitaminů A, C a E v krevní plasmě (Driskell a kol., 1982; Tanishima a Kita, 1993)

Analýza folátů v krevní plasmě (CEDIA folate kit, Roche Diagnostics)

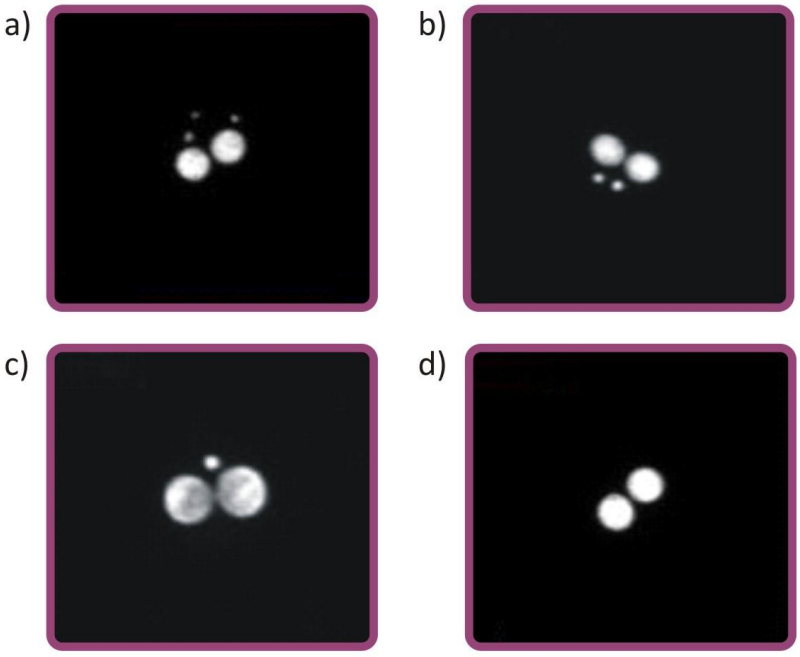
Stanovení hladin triglyceridů, celkového cholesterolu, LDL a HDL cholesterolu (diagnostic kits, Sigma)

Statistické analýzy (programy: Microsoft Excel 2000®, SAS 9.1.3, SPSS 17.0, Statistica 7.1)



Obr.7:

Celochromozomové barvení chromozomů #1 (červeně obarvený) a #4 (zeleně obarvený): a) reciproká translokace mezi barvenými chromozomy, b) inzerce chromozomu #4 do chromozomu #1 (archiv Beskid).



Obr.8:

Automatická obrazová analýza mikrojader: a) – c) DB s různým počtem MN, d) DB bez MN (archiv Rössnerová).

4. Výsledky (viz kapitola 9, přílohy 1-9)

4.1. Výsledky získané s využitím metody FISH (přílohy 1-5)

Příloha 1:

R.J. Sram, O. Beskid, **A. Rossnerova,** P. Rossner, Z. Lnenickova, A. Milcova, I. Solansky, B. Binkova: Environmental exposure to carcinogenic polycyclic aromatic hydrocarbons – the interpretation of cytogenetic analysis by FISH. *Toxicology Letters* 172 (2007) 12-20.

Příloha 2:

A. Rossnerova, I. Balascak, P. Rossner Jr., R.J. Sram: Frequency of chromosomal aberrations in Prague mothers and their newborns. *Mutation Research* 699 (2010) 29-34.

Příloha 3:

P. Rossner Jr., **A. Rossnerova**, R.J. Sram: Oxidative stress and chromosomal aberrations in an environmentally exposed population. *Mutation Research* 707 (2011) 34-41.

Příloha 4:

R.J. Sram, B. Binkova, O. Beskid, A. Milcova, P. Rossner, P. Rossner Jr., **A. Rossnerova**, I. Solansky, J. Topinka: Biomarkers of exposure and effect – interpretation in human risk assessment. *Air Quality, Atmosphere and Health 4* (2011) 161-167.

Příloha 5:

P. Rossner Jr., K. Uhlirova, O. Beskid, **A. Rossnerova**, V. Svecova, R.J. Sram: Expression of *XRCC5* in peripheral blood lymphocytes is upregulated in subjects from a heavily polluted region in the Czech Republic. *Mutation Research* 713 (2011) 76-82.

4.2. Výsledky získané s využitím automatické obrazové analýzy MN (přílohy 6-9)

Příloha 6:

A. Rossnerova, M. Spatova, P. Rossner, I. Solansky, R.J. Sram: The impact of air pollution on the level of micronuclei measured by automated image analysis. *Mutation Research* 669 (2009) 42-47.

Příloha 7:

A. Rossnerova, M. Spatova, Ch. Schunck, R.J. Sram: Automated scoring of lymphocyte micronuclei by the MetaSystems Metafer image cytometry system and its application in studies of human mutagen sensitivity and biodosimetry of genotoxin exposure. *Mutagenesis* 26 (2011) 169-175.

Příloha 8:

A. Rossnerova, M. Spatova, P. Rossner Jr., Z. Novakova, I. Solansky, R.J. Sram: Factors affecting the frequency of micronuclei in asthmatic and healthy children from Ostrava. *Mutation Research* 708 (2011) 44-49.

Příloha 9:

A. Rossnerova, M. Spatova, A. Pastorkova, N. Tabashidze, M. Veleminsky Jr., I. Balascak, I. Solansky, R.J. Sram: Micronuclei levels in mothers and their newborns from regions with different types of air pollution. *Mutation Research* 715 (2011) 72-78.

5. Diskuze souboru prací

V této práci jsou uvedeny nejnovější publikované výsledky cytogenetických analýz provedených v Oddělení genetické ekotoxikologie Ústavu experimentální medicíny AV ČR, v.v.i. Protože hodnocení vlivu k-PAU na poškození DNA bylo provedeno především s využitím dvou metod, které reprezentují stanovení různých druhů poškození, je tato práce diskutována ve dvou logických celcích. První tvoří výsledky získané s využitím celochromozomového barvení chromozomů #1 a #4, kde byly hodnoceny především stabilní chromozomální aberace ve vztahu k různým expozicím k-PAU u skupin městských strážníků, řidičů autobusů, pracovníků garáží, administrativních pracovníků, matek a jejich novorozenců z Prahy a administrativních pracovníků z Ostravy (5.1.). Druhý celek je zaměřen na analýzu mikrojader v dvoujaderných buňkách lidských periferních lymfocytů, která byla nově prováděna s využitím automatické obrazové analýzy. V rámci analýz MN byl hodnocen především vliv různých koncentrací B[a]P na studované skupiny, kterými byli městští strážníci, řidiči autobusů a administrativní pracovníci z Prahy, zdravé děti a děti trpící astmatem z Ostravy, pilotní skupina pracovníků laboratoře a matky a jejich novorozenci z Prahy a Českých Budějovic (5.2.). V závěru je pak diskutováno postavení obou metodik v biomonitorovacích studiích (5.3.) a interpretace získaných výsledků jako celku (5.4.).

5.1. Vliv k-PAU na poškození chromozomů analyzované metodou FISH (přílohy 1-5)

Analýzy vlivů k-PAU diskutovaných v první části této práce přímo navazují na závěry a zkušenosti Mgr. Oleny Beskid, Ph.D., která shrnula své poznatky z FISH analýz vzorků osob převážně profesionálně exponovaných nejrůznějším chemickým látkám ve své disertační práci (Beskid, 2006b) a zároveň se začala věnovat vlivům karcinogenních znečišťujících látek v ovzduší. Zde diskutovaný soubor prací zahrnuje v první publikaci opakované hodnocení 61 městských strážníků z Prahy v obdobích leden a březen 2004 (příloha 1), kde byly studovány kromě vlivu k-PAU na frekvence translokací i vlivy dalších faktorů. Druhý rukopis se věnuje hodnocení stabilních a nestabilních chromozomálních aberací u skupiny 42 matek a 55 novorozenců z Prahy v období podzim/zima 2007/2008 (příloha 2). Další rukopis diskutuje

souvislosti mezi oxidačním poškozením a chromozomálními aberacemi u skupiny 59 městských strážníků z Prahy sledovaných v únoru a květnu 2007 (příloha 3). Souhrnná analýza vlivu expozice B[a]P na genomickou frekvenci translokací provedená celkem u 950 jedinců sledovaných v letech 2001-2006 (příloha 4) je předmětem dalšího rukopisu. Poslední příloha tohoto celku přináší nejnovější poznatky z porovnání 64 osob z Prahy a 75 osob z Ostravy v zimním období roku 2010 (příloha 5). Celkový diskutovaný soubor v této části práce představuje 1304 osob, z nichž některé byly hodnoceny opakovaně v různých obdobích.

5.1.1. Využití analýzy FISH pro hodnocení osob exponovaných k-PAU (příloha 1)

Vzhledem k tomu, že Praha patří v rámci České republiky mezi nejvíce znečištěné oblasti, kde žije zhruba 12% obyvatel, byla řada studií včetně této prováděna právě zde. Studie z roku 2004 porovnává opakovaně v lednu a březnu frekvence chromozomálních aberací u skupiny 61 městských strážníků pracujících v ulicích centra města. Monitoring znečištění ovzduší byl proveden s využitím stacionárního monitoru VAPS (Versatile Air Pollution Sampler) a pro zjištění osobní zátěže každého účastníka studie byly navíc využity personální monitory. Při porovnání studovaných období byl zjištěn významný pokles koncentrací všech studovaných znečišťujících látek (PM10, PM2,5, k-PAU a B[a]P) měřených stacionárním monitoringem, stejně tak jako k-PAU a B[a]P podle personálního monitoringu (k-PAU: 9,07±9,94 ng/m^3 vs. 3,46±4,65 ng/m^3, p<0,001; B[a]P: 1,58±1,39 ng/m^3 vs. 0,39±0,64 ng/m^3, p<0,001) pro leden vs. březen. Zjištěné hodnoty genomické frekvence translokací se rovněž signifikantně lišily (F_G/100 = 1,32±1,07 pro leden vs. 0,85±0,95 pro březen, p<0,001). V této práci bylo rovněž provedeno porovnání citlivosti metodik FISH a CCA. Byl zde rovněž studován možný vliv celé řady dalších faktorů (věku, kotininu v moči, hladin vitaminů A, C a E v krevní plasmě, folátů, celkového, HDL a LDL cholesterolu a triglyceridů) na frekvence chromozomálních aberací.

Hlavním poznatkem z této studie byl signifikantní pokles hladiny stabilních chromozomálních aberací v souvislosti s významným poklesem koncentrací studovaných znečišťujících látek. Již dříve byla pozorována podobná situace u skupiny pracovníků

exponovaných ethylbenzenu, kde rovněž došlo k významnému poklesu genomické frekvence translokací během několika měsíců (Sram a kol., 2004b). Pro CCA bylo odhadováno, že zjištěná frekvence chromozomálních aberací odráží expozici chemickým karcinogenům až tři měsíce před odběrem PBL (Sram a kol., 2004a). Naproti tomu byly stabilní aberace hodnocené metodou FISH považovány za aberace, které odráží expozici za několik let (Kleinerman a kol., 2006), což má význam zejména pro hodnocení vlivu ozáření (Edwards a kol., 2005). V jiné studii provedené na krysách po jejich ozáření dávkou 1 a 2 Gy bylo však ukázáno, že frekvence translokací klesá již 4 dny po expozici (Tucker a kol., 1997). Podobné závěry byly publikovány po ozáření PBL in vitro dávkami 0,2-4,0 Gy, kde frekvence translokací klesla během sedmi dnů (Tucker a kol., 2005a; Tucker a kol., 2005b). Naše studie ukázala na signifikantní změny v hladinách translokací již po několika týdnech a zároveň na vhodnost metodiky FISH jako citlivého biomarkeru pro hodnocení rizika expozice k-PAU. Naproti tomu výsledky získané z CCA ukázaly sice na pokles %AB.B. (procento aberantních buněk), nicméně tato změna nebyla signifikantní pravděpodobně i kvůli nižšímu počtu hodnocených metafází v porovnání s metodikou FISH, kde jich je hodnoceno 10x více. Podobně jako v další publikované práci (Sram a kol., 2007a) ukazuje zmíněné srovnání na vyšší citlivost metodiky FISH pro tento druh studií.

V rámci hodnocení dalších faktorů majících vliv na genomickou frekvenci translokací byl patrný především efekt věku, což je v souladu s publikovanými daty (Sigurdson a kol., 2008). Vliv zvýšené hladiny celkového a LDL-cholesterolu byl na hranici signifikance stejně jako i vliv nedostatečného příjmu vitaminu C. Komplexnější hodnocení vlivu výživy bylo studováno ve vztahu ke vzniku MN (Fenech a kol., 2005), podobná studie ve vztahu ke stabilním translokacím je výzvou pro další výzkum.

V souhrnu bylo v této studii s využitím metodiky FISH především naznačeno zvýšené genotoxické riziko pro skupinu pražských městských strážníků při expozicích > 1 ng B[a]P/m^3. Rovněž zde byla poprvé ukázána změna frekvence translokací indukovaná k-PAU v průběhu několika týdnů. FISH se tak stala vedle metodiky k hodnocení rizika profesionálních expozic chemickým látkám i metodikou pro hodnocení vlivu environmentálních expozic.

5.1.2. Frekvence stabilních a nestabilních aberací u matek a jejich novorozenců (příloha 2)

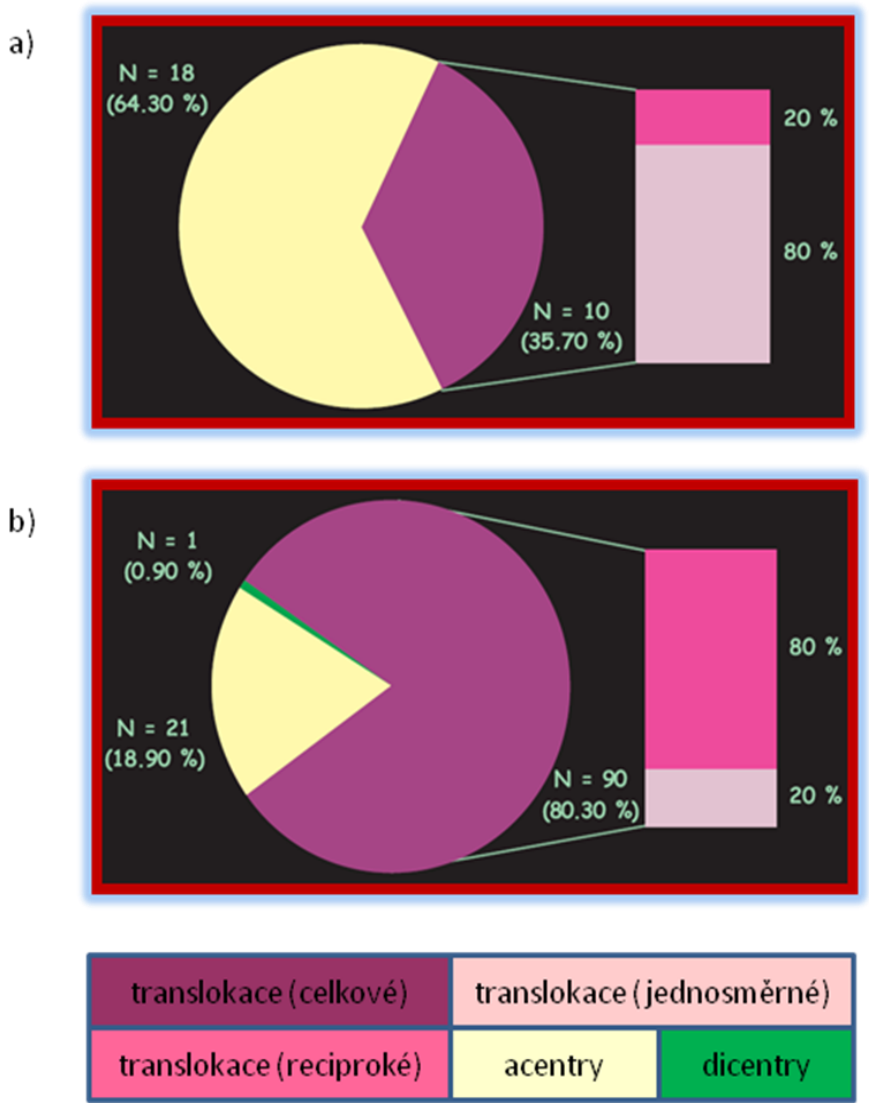
Další studie vlivu k-PAU na frekvence chromozomálních aberací studovaných metodou FISH byla provedena u skupin pražských matek a jejich novorozenců v období říjen 2007 až únor 2008, kdy koncentrace těchto látek v ovzduší dosahují nejvyšších hodnot. Průměrné hodnoty k-PAU a B[a]P byly podle stacionárního monitoringu v lokalitě Praha-Smíchov pro hodnocené období 21,0±12,3 ng/m^3 pro k-PAU a 2,9±1,8 ng/m^3 pro B[a]P. Hodnocená skupina zahrnovala 42 matek s průměrným věkem 29 let a jejich novorozenců. Ve studii jsme kromě vlivu k-PAU hodnotili zastoupení stabilních chromozomálních aberací reprezentovaných především jednosměrnými (one-way) a reciprokými (two-way) translokacemi a zastoupení nestabilních aberací jako jsou acentrické fragmenty. Byl rovněž analyzován vliv věku matek na frekvence chromozomálních aberací novozenců.

V naší studii jsme porovnávali vliv koncentrací k-PAU rozdělených podle mediánu na frekvenci jednotlivých druhů aberací. Vzhledem k velmi úzkému koncentračnímu rozpětí v průběhu studovaného období nebyl pozorován žádný efekt na genetické poškození ani u jedné ze studovaných skupin. Vliv expozice k-PAU na frekvence stabilních a nestabilních chromozomálních aberací u skupiny novorozenců byl hodnocen ve studiích provedených v New Yorku (Bocskay a kol., 2005; Bocskay a kol., 2007). Bocskay a kol. publikovali signifikantní vliv prenatální expozice k-PAU na hladinu stabilních aberací u skupiny 60 novorozenců afroamerického a dominikánského původu. Výsledek této studie je při porovnání s naší prací překvapivý, neboť při výrazně nižší expozici v New Yorku byla naopak frekvence stabilních aberací u novorozenců několikanásobně vyšší. Tento výrazný rozdíl si vysvětlujeme jiným způsobem hodnocení chromozomálních aberací než je obvyklé podle protokolu PAINT (Tucker a kol., 1995) a zahrnutím delecí mezi stabilní aberace. Rovněž stravovací návyky této nízkopříjmové skupiny mohly přispět k tak rozdílným výsledkům. Naše hodnota genomické frekvence translokací u novorozenců (F_G/100 = 0,09) je v souladu s daty publikovanými v jiných studiích (Pluth a kol., 2000; Sigurdson a kol., 2008). Pluth a kol. uvádí hodnotu F_G/100 = 0,11 pro novorozence nekuřaček, které byly vybrány i do naší studie, a Sigurdson a kol. publikuje hodnotu F_G/100 = 0,04, která byla zjištěna v rámci mezinárodní studie, kde bylo hodnoceno celkem 296 novorozenců.

Hodnocení vlivu věku na frekvence stabilních a nestabilních chromozomálních aberací u novorozenců a matek ukázalo v naší studii na devítinásobné zvýšení stabilních aberací a pouze minimální zvýšení u acentrických fragmentů. Vliv věku na akumulaci cytogenetického poškození byl již dříve studován (Ramsey a kol., 1995). V citované studii byly porovnávány frekvence stabilních a nestabilních aberací mezi novorozenci a dospělými ve věku 19-79 let. Podobně jako v naší studii pozorovali autoři 10,6ti násobný nárůst frekvence stabilních aberací a podstatně nižší zvýšení pro nestabilní aberace. Naše výsledky rovněž přinášejí údaje o změně spektra aberací pro obě sledované skupiny. Zatímco u novorozenců připadalo 64,3% na nestabilní a 35,7% na stabilní aberace, u matek byl tento poměr výrazně odlišný a nestabilní aberace zahrnující acentrické fragmenty a dicentrické chromozomy představovaly pouze 19,7%, vedle dominujících stabilních, které tvořily 80,3% všech zjištěných případů (obrázek 9). Podobný poměr stabilních a nestabilních aberací jako u matek v naší studii byl již dříve publikován pro skupinu 49 mužů z Prahy se stejným průměrným věkem 29 let (Beskid a kol., 2006a).

V naší studii byl zjištěn signifikantní nárůst stabilních aberací u novorozenců ($p<0,05$), kteří se narodili starším matkám ve věku 31-40 let v porovnání s novorozenci mladších matek (věk 20-30 let). Vliv věku matek na zvýšené riziko spontánních potratů a vážných chromozomálních abnormalit u jejich plodu je dobře znám. V této studii jsme upozornili na skutečnost, že frekvence stabilních aberací u zdravého novorozence může být rovněž ovlivněna věkem matky a může být příčinou zdravotních problémů v pozdějším věku. Na rozdíl od stabilních aberací se zdá, že věk matek nesouvisí s nárůstem frekvence méně závažných nestabilních aberací u jejich novorozenců.

V této studii jsme pozorovali zřetelné rozdíly v zastoupení různých typů chromozomálních aberací u matek a jejich novorozenců, které byly dány především věkem. Rovněž byl pozorován vliv věku matek na zastoupení chromozomálních aberací u novorozenců. Vzhledem k tomu, že nebyl pozorován vliv znečištěného ovzduší kvůli malým rozdílům v koncentracích k-PAU v průběhu studovaného období, zaměřili jsme se v další studii na vlivy znečišťujících látek v ovzduší ve dvou rozdílných lokalitách (příloha 9).



Obr.9:

Poměrné zastoupení jednotlivých druhů chromozomálních aberací: a) novorozenci, b) matky.

5.1.3. Oxidační poškození a chromozomální aberace v environmentálně exponované populaci (příloha 3)

Studiem souvislostí mezi chromozomálními aberacemi a oxidačním poškozením u skupiny městských strážníků se zabývá následující práce, kde byl zároveň monitorován efekt expozice k-PAU, včetně B[a]P, a efekt expozice PM2,5. Na základě již dříve zjištěné sezonní variability (příloha 1) byla studovaná skupina 59 městských strážníků z Prahy

monitorována opakovaně, a to v únoru a v květnu 2007. Práce se rovněž podrobně věnuje vlivu studovaných znečišťujících látek na zmíněné biomarkery ve vztahu k různým časovým intervalům expozice znečišťujícím látkám před vlastním odběrem biologického materiálu.

Údaje o znečištění ovzduší ze stacionárního monitoringu ukázaly při porovnání obou studovaných období na signifikantní pokles v koncentracích k-PAU a B[a]P ($p<0,001$). Stejný trend byl zaznamenán pro personální expozice těmito znečišťujícími látkami ($p<0,001$). Narozdíl od těchto výsledků měly expozice prachovým částicím PM2,5 opačný trend a podle údajů z ČHMÚ byly koncentrace signifikantně vyšší v květnu 2007 ($p<0,001$). Vedle již zmíněného zařazení B[a]P mezi látky karcinogenní pro člověka (IARC, http://monographs.iarc.fr) jsou PM2,5 podle nedávno publikovaného review (Chen a kol., 2008) vážným rizikovým faktorem zvyšování mortality obyvatelstva (každý nárůst koncentrace o 10 $\mu g/m^3$ zvyšuje mortalitu o 6% bez ohledu na věk, pohlaví či lokalitu). Protisměrný trend těchto klíčových expozic byl pravděpodobně hlavním důvodem, proč nebyl pozorován signifikantní rozdíl pro žádný ze studovaných cytogenetických markerů ($F_G/100$, %AB.B. a acentrických fragmentů), ani markerů oxidačního poškození biomolekul (pro DNA – 8-oxodG, pro lipidy – 15-F2t-IsoP a pro proteiny – hladina karbonylových skupin).

Analýza asociace mezi cytogenetickými markery a markery oxidačního stresu ukázala na negativní vztah mezi oxidačním poškozením proteinů a $F_G/100$, stejně tak jako mezi oxidačním poškozením proteinů a frekvencí acentrických fragmentů. Zvýšené oxidační poškození proteinu bylo asociované s protektivním účinkem na frekvenci chromozomálních aberací, což představuje zcela nový poznatek. Tento jev může být dáván do souvislosti s antioxidačními vlastnostmi albuminu, který je zároveň nejzastoupenějším proteinem v krevní plasmě (Roche a kol., 2008). Je známo, že hydroxylové radikály mají vliv na fragmentaci proteinů a zvýšení hladiny karbonylových skupin (Davies a Delsignore, 1987), nicméně na základě našich výsledků můžeme o albuminu uvažovat jako o filtru na hydroxylové radikály, které by jinak poškozovaly DNA a vedly ke zvýšené frekvenci zlomů.

V naší studii byl navíc podle údajů ze stacionárního monitoringu analyzován vliv studovaných znečišťujících látek na jednotlivé biomarkery u celé skupiny, bez ohledu na období odběru. V časových modelech byl hodnocen vliv expozic v intervalech 3, 14 a 28 dní až do tří měsíců před odběrem. Podle získaných výsledků byla frekvence aberantních buněk

hodnocená metodou FISH ovlivněná expozicemi B[a]P v období 57-70 dní před odběrem biologického materiálu, což je v souladu se studií z roku 2009, kde byl zjištěn vliv k-PAU na frekvenci MN až 60 dní před odběrem vzorků (Rossnerova a kol., 2009). Výsledky vlivů znečišťujících látek ve vztahu k oxidačnímu poškození jsou rovněž v souladu s předchozími analýzami, kde PM2,5 měly rychlý účinek (3-denní perioda) na zvýšení hladiny 8-oxodG (Rossner Jr. a kol., 2008a) stejně jako B[a]P na 15-F2t-IsoP (Rossner Jr. a kol., 2008b). Další výsledky ukázaly, že perioda 57-84 dní před odběrem souvisela se zvýšeným vylučováním 8-oxodG v moči indukovaného hladinou B[a]P. Z uvedených výsledků vyplývá vedle zmíněného okamžitého efektu PM2,5 na oxidační poškození DNA, podstatně dlouhodobější vliv B[a]P, který pravděpodobně souvisí s jeho alternativní metabolickou dráhou, při které vzniká o-chinon. Z tohoto důvodu je k detekci indukované zvýšené hladiny 8-oxodG v moči nutný delší časový interval.

Kromě již diskutovaných výsledků byla ve studii provedena i analýza věku a faktorů životního stylu (vliv kotininu, LDL, HDL a celkového cholesterolu a triglyceridů) na studované biomarkery. Signifikantní vliv věku na frekvenci stabilních chromozomálních aberací byl v souladu s literaturou (Sigurdson a kol., 2008). Z faktorů životního stylu byl jediným signifikantním výsledkem vliv zvýšené hladiny cholesterolu na F_G/100, což bylo v souladu s naší předchozí studií (příloha 1).

Práce přinesla zejména nové poznatky o významném vlivu PM2,5 na studované biomarkery v období, kdy byla koncentrace dalších studovaných znečišťujících látek signifikantně nižší, a zároveň nové informace o časovém dosahu vlivu studovaných látek na analyzované biomarkery. Ve studii byl diskutován možný protektivní efekt zvýšeného oxidačního poškození proteinů na formování chromozomálních aberací.

5.1.4. Biomarkery expozice a účinku – interpretace pro hodnocení rizika expozice k-PAU (příloha 4)

Interpretací biomarkerů expozice a účinku ve vztahu k hodnocení rizika pro lidské zdraví se zabývá další práce, která analyzuje data ze tří studií provedených v letech 2001 – 2006. Hodnoceno bylo celkem 950 osob z Prahy, převážně městských strážníků a kontrol (roky

2001 a 2004) a řidičů autobusů, pracovníků garáží a kontrol (roky 2005 – 2006). Hlavním cílem bylo vyhodnotit u sledovaných osob efekt expozice k-PAU měřených s využitím personálního monitoringu na frekvenci chromozomálních aberací a hladinu aduktů DNA. Pro hodnocení chromozomálních aberací byla využita metodika FISH a CCA a pro stanovení aduktů DNA metoda ^{32}P-postlabeling. Mezi dalšími provedenými analýzami v jednotlivých studiích bylo stanovení kotininu v moči (ng kotininu/mg kreatininu), vitaminů A, C a E a folátů v krevní plasmě (µmol/l), stanovení hladin triglyceridů, celkového cholesterolu, LDL a HDL cholesterolu (mmol/l) a stanovení polymorfismů metabolických a reparačních genů.

Výsledky personálního monitoringu 53 městských strážníků (29 kuřáků + 24 nekuřáků) a 52 kontrol (11 kuřáků + 41 nekuřáků) sledovaných v únoru roku 2001 ukázaly na signifikantně vyšší expozici městských strážníků oproti kontrolám, jak pro k-PAU (9,7 ng/m^3 vs. 5,8 ng/m^3, p<0,01), tak pro B[a]P (1,6 ng/m^3 vs. 0,8 ng/m^3, p<0,01). Signifikantní rozdíly ve stejném trendu byly zaznamenány rovněž pro genomickou frekvenci translokací (F_G/100 exponovaných vs. kontrol bylo 1,72±1,57 vs. 1,24±1,11, p<0,05) a pro hladinu "like-B[a]P"-DNA aduktů (adukty indukované B[a]P) (0,122±0,036 vs. 0,099±0,035 aduktů/10^8 nukleotidů, p=0,003). Výsledky ukázaly na asociaci frekvence translokací s věkem, kouřením, hladinou folátů, polymorfismy v genech *CYP1A1*2 C, GSTP1, EPHX1, p53, MTHFR* a "like-B[a]P"-DNA adukty. Ve vztahu k celkovým aduktům DNA byl vyhodnocen signifikantní vliv kouření, hladiny vitaminu C a polymorfismu v genu *XPD* v exonu 23 a genu *GSTM1*. Hladina "like-B[a]P"-DNA aduktů byla ovlivněna především expozicí znečišťujícím látkám z ovzduší, kouřením, a polymorfismem v reparačním genu *XPD* v exonu 6 (Binkova a kol., 2007). Z této části studie vyplynulo, že celkové adukty DNA, "like-B[a]P"-DNA adukty a genomická frekvence translokací byly ovlivněny kouřením. Efekt expozice znečištěnému ovzduší je vhodné analyzovat u skupin nekuřáků, jak již bylo dříve navrženo (Binkova a kol. 2007; Sram a kol. 2007a).

Opakované monitorování městských strážníků v lednu, březnu, červnu a září bylo již ve vztahu k analýze FISH a CCA částečně popsáno v příloze 1. Koncentrace k-PAU vykazovaly sezonní variabilitu a pro uvedené pořadí měsíců byly podle personálního monitoringu 9,07 ng/m^3, 3,46 ng/m^3, 1,92 ng/m^3 a 3,08 ng/m^3 a pro B[a]P 1,58 ng/m^3, 0,39 ng/m^3, 0,18 ng/m^3 a 0,45 ng/m^3. Tak, jako byl popsán signifikantní pokles genomické frekvence translokací,

došlo i zde k signifikantnímu poklesu “like-B[a]P“-DNA aduktů při porovnání měsíců leden a březen ($p<0,001$) a leden a červen ($p=0,017$). Kromě sezonní variability frekvence stabilních chromozomálních aberací indikují výsledky této části souhrnné studie závěr, že expozice k-PAU hraje pravděpodobně klíčovou roli při formování aduktů DNA v PBL (Topinka a kol., 2007).

Další hodnocené skupiny, které se staly součástí souhrnné analýzy vlivu sledovaných znečišťujících látek, byly pražské skupiny řidičů autobusů, pracovníků garáží a administrativních pracovníků, sledované opakovaně v zimě 2005, létě 2006 a zimě 2006. Průměrné hodnoty a směrodatné odchylky $F_G/100$ pro uvedené pořadí odběrů pro skupinu řidičů byly 1,62±1,17, 2,18±1,75 a 1,77±1,31; pro skupinu pracovníků garáží 1,20±1,24, 0,88±1,11 a 1,01±0,78; pro administrativní pracovníky 1,65±1,49, 1,34±1,01 a 1,87±1,29. Uvedené výsledky genotoxického poškození u kontrolní skupiny a pracovníků garáží byly v souladu s dříve publikovanou sezonní variabilitou (Sram a kol., 2007b), zatímco skupina řidičů představovala z hlediska expozice unikátní skupinu, kde se předpokládá vzhledem k charakteru zaměstnání v letním období zvýšený kontakt s vnějším prostředím. Nižší frekvence genotoxického poškození u pracovníků garáží souvisí s nižším věkovým průměrem této skupiny (Sigurdson a kol., 2008). Analýza aduktů DNA byla u těchto skupin rovněž provedena a zahrnuta do souhrnné analýzy.

V souhrnné analýze provedené s využitím multivariátní regrese pro oba testované biomarkery byl zjištěn signifikantní vztah mezi personální expozicí B[a]P a genomickou frekvencí translokací ($F_G/100=1,255+B[a]P \times 0,082$, $p<0,05$), stejně tak jako mezi personální expozicí B[a]P a hladinou aduktů DNA (DNA adukty$=1,042+B[a]P \times 0,077$, $p<0,001$). Oba studované biomarkery byly již dříve analyzovány ve vztahu ke zvýšenému riziku některých onemocnění. Zvýšená frekvence chromozomálních aberací v PBL se stala prediktivním faktorem rizika nádorových onemocnění (Bonassi a kol., 2008), stejně tak jako formování aduktů DNA, které je rovněž považováno za klíčový krok v karcinogenezi (Gammon a kol., 2004; Phillips, 2005). Jiná studie poukazuje na signifikantní souvislosti mezi hladinou aduktů DNA a aterosklerózou (Binkova a kol., 2002).

Tato práce ukázala zejména na vhodnost použitých biomarkerů pro hodnocení rizika expozice studovaným karcinogenům a zároveň poprvé poukazuje na souvislosti mezi adukty

DNA (biomarker expozice) a chromozomálními aberacemi hodnocenými metodou FISH (biomarker účinku). Uvedené výsledky, společně s našimi dalšími studiemi (Topinka a kol., 2007; Sram a kol., 2007a; Sram a kol., 2007b; Rossnerova a kol., 2009a; Rubes a kol., 2010) ukazují, že koncentrace B[a]P > 1 ng/m^3 indukuje poškození DNA. Citované práce se staly podkladem pro závěry WHO (WHO, 2010).

5.1.5. Hladiny biomarkerů a exprese reparačních genů v různě znečištěných oblastech (příloha 5)

Nejnovější studie vztahující se k frekvenci stabilních chromozomálních aberací studovaných metodou FISH porovnává úroveň poškození mezi lokalitami, které se významně liší úrovní znečištění. Ve studii bylo v zimním období roku 2010 analyzováno 64 osob z Prahy a 75 osob z Ostravy. Průměrné personální expozice účastníků studie všem studovaným znečišťujícím látkám byly signifikantně vyšší ($p<0,001$) v Ostravě (k-PAU: 118,7±117,2 ng/m^3; B[a]P: 17,09±16,85 ng/m^3; benzen: 17,81±11,78 µg/m^3) oproti Praze (k-PAU: 19,60±14,56 ng/m^3; B[a]P: 2,81±1,88 ng/m^3; benzen: 5,64±1,68 µg/m^3). Ve studii byly kromě cytogenetických markerů hodnoceny rovněž markery oxidačního stresu a faktory životního stylu. Nově byly navíc porovnávány úrovně exprese vybraných reparačních genů, které mají vztah k bázové excizní reparaci (BER) (*OGG1*, *APEX1*, *XRCC1*) a nehomologní rekombinaci (NHEJ) (*LIG4*, *XRCC4*, *XRCC5*, *XRCC6*). Výběr reparačních enzymů byl dán studovanou reparační dráhou, kde enzymy BER hrají významnou roli v opravě oxidovaných bází (Powell a kol., 2005), zatímco enzymy NHEJ mají význam při opravách chromozomálních zlomů (Lieber a kol., 2006).

Přesto, že expozice k-PAU, B[a]P a benzenu byly statisticky výrazně vyšší v Ostravě, hladina analyzovaných biomarkerů těmto trendům neodpovídala. Cytogenetické markery, oxidační poškození biomolekul, ani exprese vybraných genů se od sebe nelišily. Skupiny se však významně lišily příjmem vitaminů A, C a E a hladinou HDL cholesterolu, které byly signifikantně vyšší ($p<0,001$) u ostravské skupiny. Významným pozitivem pro tuto skupinu byla i signifikantně nižší ($p<0,001$) expozice environmentálnímu tabákovému kouři (ETS). Protektivní vliv na nižší oxidační poškození DNA měla v celé skupině na základě multivariátní

analýzy vyšší hladina vitaminů C a E v krevní plasmě. Obdobně vyšel i vztah mezi peroxidací lipidů a vitaminem C. Ve vztahu k reparačním enzymům byla vyšší hladina 8-oxodG asociována se signifikantně vyšší expresí genu *XRCC1*. U cytogenetických markerů F_G/100 a %AB.B. byl patrný vliv věku ($p<0,001$), což odpovídá publikovaným trendům (Sigurdson a kol., 2008), a naznačen byl rovněž vliv benzenu. Výsledky bivariátní a multivariátní analýzy asociace mezi genovou expresí a lokalitou ukázaly na signifikantně zvýšenou expresi genu *XRCC5* u ostravských účastníků studie, a to i při zavedení korekce (q) na mnohonásobné porovnávání (OR, 95% CI: 3,33, 1,03-10,8, q=0,046).

Frekvence chromozomálních aberací hodnocená metodou FISH byla již analyzována v celé řadě našich předchozích studiích (Beskid a kol., 2007; Sram a kol., 2007a; Sram a kol. 2007b; Rossner Jr. a kol. 2011), které byly provedeny při průměrných individuálních expozicích B[a]P 0,2-3,8 ng/m^3. Žádná z prací však nebyla prováděná při tak vysokých průměrných personálních expozicích B[a]P 17,09 ng/m^3 (maximum 74,2 ng/m^3), jako tato. Na rozdíl od předchozích studií však nebyl s využitím našich biomarkerů zjištěn předpokládaný vztah mezi dávkou a účinkem.

Na základě dalších analyzovaných faktorů se nabízí několik vysvětlení. První podstatný rozdíl vyplývající z našich analýz souvisí se signifikantním rozdílem mezi oběma lokalitami v expresi genu *XRCC5*, jehož proteinový produkt XRCC5 (Ku80) vytváří společně s XRCC6 (Ku70) heterodimerní komplex Ku, který je zároveň prvním proteinem, který hraje významnou roli v NHEJ při rozpoznávání dvouřetězcových zlomů DNA, na jejichž konce se váže. V literatuře byl popsán vztah mezi zvýšenou expresí Ku komplexu a sníženou reparační kapacitou dvouřetězcových zlomů (Kasten a kol., 1999), což by zároveň mohlo vysvětlovat nižší frekvenci stabilních translokací u ostravské skupiny. Jiné vysvětlení redukce translokací by mohlo souviset s přesností NHEJ, ve které hraje významnou roli protein BRCA1 (Durant a Nickoloff, 2005). Tento protein se podílí na bezchybném průběhu NHEJ tím, že potlačuje aktivitu komplexu MRN, který sestává z proteinů RAD50/MRE11/NBS1. Komplex MRN odstraňuje sekvence přesahující dvouřetězcové zlomy v DNA, čímž způsobí vznik chyb při NHEJ. Můžeme se domnívat, že u ostravských subjektů byl preferenčně aktivován mechanismus přesného NHEJ vedoucího k opravě DNA bez indukce chromozomálních

aberací. Obě zmíněné teorie však neřeší, proč nebyl zjištěn rozdíl v nestabilních aberacích reprezentovaných acentrickými fragmenty.

Dalším analyzovaným signifikantním rozdílem byl již zmíněný vyšší příjem vitaminů A, C a E. Je známo, že vitaminy C a E jsou významnými antioxidanty, zatímco role vitaminu A je v tomto ohledu méně významná (Dragsted, 2008). Negativní asociace mezi koncentrací vitaminů C a E a hladinou vyloučeného 8-oxodG analyzována v této studii podporuje hypotézu, že antioxidační vlastnosti těchto vitaminů snižují formování 8-oxodG. Jiným aspektem, který mohl hrát roli ve snížení hladin oxidačního stresu v Ostravě, mohl být efekt vitaminů na expresi jednotlivých genů. Naše data naznačují, že *XRCC1* by mohl být jedním z těchto genů. Jeho proteinový produkt XRCC1 hraje významnou roli v interakcích s většinou komponentů dráhy „short patch" BER (Almeida a Sobol, 2007), kterými jsou další studované proteiny (OGG1 a APEX1). Na základě toho se můžeme domnívat, že zvýšená exprese genu *XRCC1* asociovaná s hladinou vitaminu C vyšší než medián má ve svém důsledku vliv na rychlejší odstraňování 8-oxodG z DNA a vyšší reparační kapacitu.

Tato práce je unikátní především tím, že hodnotí hladiny biomarkerů v nejznečištěnější části naší republiky, která je zároveň jedinečná v rámci celé Evropské unie. Překvapivě však měly osoby žijící v silně znečištěné Ostravě srovnatelné hladiny biomarkerů jako osoby z podstatně čistší Prahy. Vysvětlením je nejspíše adaptace organismu daná změnami v profilech genové exprese. Již dříve byly publikovány údaje o odlišné úrovni genové exprese mezi různými lokalitami (Leeuwen a kol., 2006). Naše práce ukázala na význam zvýšené exprese genu *XRCC5* a zvýšené hladiny vitaminů C a E při kompenzaci negativních efektů znečištěného ovzduší na lidské zdraví.

Na závěr tohoto tématického bloku (*5.1.*) je třeba ještě upozornit na skutečnost, že část vzorků analyzovaných metodou FISH byla hodnocena po manuálním vyhledávání metafází (přílohy 1, 4 a 5), zatímco další část (novorozenci a matky a skupina strážníků hodnocená opakovaně v roce 2007) byla hodnocena po automatickém vyhledávání (přílohy 2 a 3). Jak již bylo zmíněno v úvodu, není plná automatizace zatím možná a hodnocení konkrétních typů aberací je stále závislé na lidském faktoru (Tucker, 2010). Z našich zkušeností vyplývá, že

automatické vyhledávání metafází s využitím programů MSearch a jejich následné zvětšování v plném barevném rozlišení je pro další hodnocení, které spočívá v kontrole galerií nasnímaných obrázků, pohodlnější, přináší rovnocenné výsledky, nicméně vzhledem k množství technických manipulací nepřináší výraznější časovou úsporu při hodnocení, ale pouze alternativu pro část postupu.

5.2. Využití automatické obrazové analýzy MN v biomonitorovacích studiích (přílohy 6-9)

Na rozdíl od studií využívajících k hodnocení FISH byly veškeré analýzy MN provedeny s využitím automatické obrazové analýzy. Využívaný program MSearch umožnil kromě nasnímání veškerých hodnocených objektů rovněž jejich analýzu. Z publikovaných prací, které jsou zde zároveň diskutovány, byla první analýza provedena u skupiny 56 městských strážníků z Prahy, kteří byli sledováni opakovaně v únoru a květnu 2007 (příloha 6). Další rukopis (příloha 7) shrnuje kromě této skupiny ještě výsledky analýz 50 řidičů autobusů a 50 administrativních pracovníků z Prahy odebraných již v listopadu 2006, hlavní výsledky ze studie věnované 81 astmatickým a 94 zdravým dětem z Ostravy, které byly studovány v listopadu roku 2008, opakované odběry u skupiny 61 a 65 městských strážníků z Prahy sledovaných vždy v únoru v letech 2009 a 2010 a dále skupinu osmi laboratorních pracovníků, z nichž čtyři v lednu 2010 strávili 3 týdny v Ostravě. Třetí rukopis (příloha 8) se podrobně věnuje již zmíněným astmatickým dětem a zdravým dětem z Ostravy. Poslední z této řady (příloha 9) je rukopis věnovaný 178 novorozencům a jejich matkám z Prahy a z Českých Budějovic, které jsme studovali po odběru v zimním období 2008/2009. Celkový publikovaný soubor dnes zahrnuje 885 analýz, přičemž některé osoby byly hodnoceny opakovaně v různých obdobích.

5.2.1. Vliv znečištěného ovzduší na frekvenci MN (příloha 6)

Skupina 56 městských strážníků z Prahy studovaná opakovaně v únoru a květnu roku 2007 se stala naší první skupinou, kde byla frekvence MN analyzována s využitím automatické obrazové analýzy (MetaSystems Metafer 4, verze 3.2.1). Práce se zároveň stala vůbec první publikovanou biomonitorovací studií s využitím tohoto systému. Hlavním cílem

bylo vyhodnocení vlivu k-PAU, zejména B[a]P, a dále VOC na frekvenci MN. Dalším cílem pak bylo vyhodnotit efektivnost automatické obrazové analýzy pro další použití v biomonitorovacích studiích.

Zprovoznění systému v naší laboratoři vyšlo především ze zkušeností publikovaných ve dvou pracích v roce 2004 (Schunck a kol., 2004; Varga a kol., 2004). První z nich obecně představila systém včetně jeho dalších aplikací na základě příslušného programového vybavení, které může kromě analýzy MN umožnit například již zmíněné vyhledávání metafází, analýzu dicentrických chromozomů, aplikaci pro kometový test atd. Druhá práce přinesla konkrétní informace o podmínkách skenování, které se týkalo zejména správného nastavení použitých klasifikátorů pro vyhledávání DB a MN. Testy funkčnosti systému, jejichž výsledkem bylo mimo jiné i doporučení optimálního barvení, kterým se stalo fluorescenční barvení DAPI, byly prováděny na PBL pacientek s nádorem prsu a kontrolách, které byly ozařovány až 2 Gy záření γ. Rovněž ve všech našich studiích bylo použito barvení DAPI, které se i nám ukázalo pro práci automatického systému jako optimální. Nastavení klasifikátorů pro analýzu DB a MN bylo optimalizováno na základě doporučení firmy MetaSystems. Stejné klasifikátory byly pak používány ve všech dalších studiích.

Do studie bylo zahrnuto 56 městských strážníků (20 kuřáků a 36 nekuřáků) s průměrným věkem 34 let. Odběr biologického materiálu a personální monitoring byl proveden opakovaně v únoru a květnu 2007. Údaje o individuálních expozicích k-PAU (benz[a]antracen, benzo[b]fluoranthen, benzo[k]fluoranthen, benzo[ghi]perylen, benzo[a]pyrene, chrysen, dibenz[ah]antrracen a indeno[cd]pyren), B[a]P a VOC (benzen, toluen, ethylbenzen a o-, m- a p-xylen) byly doplněny podle dostupnosti údajů ze stacionárního monitoringu z měřící stanice Praha-Smíchov. Doplňující údaj o hladině kotininu v moči byl využit ke kontrole údajů o kouření získaných z dotazníků. Naměřená hladina vyšší než 500 ng kotininu na 1 mg kreatininu byla spojována s aktivním kuřáctvím.

Výsledky této studie ukázaly na signifikantní pokles hladiny MN při porovnání obou období. Naměřené průměrné frekvence MN/1000 DB a směrodatné odchylky pro celou sledovanou skupinu byly následující: 7,32±3,42 pro únor 2007 a 4,67±2,92 pro květen 2007, $p<0,001$. Rozdíly ve frekvencích MN jsou v souladu se studiemi porovnávajícími frekvence MN v různě znečištěných oblastech (Ishikawa a kol., 2006b; Leeuwen a kol., 2008). Vysoce

signifikantní pokles ($p<0,001$) byl podle údajů z obou monitoringů zaznamenán u koncentrací k-PAU, B[a]P i benzenu. Vzhledem k tomu, že expozice k-PAU byly v roce 2007 nižší (personální expozice B[a]P v únoru 2007 byla pouze 1,04±0,76 ng/m^3) v porovnání s koncentracemi naměřenými v únoru 2001 (Binkova a kol., 2007), či lednu 2004 (Sram a kol., 2007b) a pokles genetického poškození byl vysoce signifikantní, bylo hlavním závěrem z této studie konstatování, že automatická obrazová analýza je dostatečně citlivá pro aplikaci v biomonitorovacích studiích. Na sumárním souboru 112 hodnocení byla navíc provedena bivariátní i multivariátní logistické regrese vlivu expozic k-PAU na naměřenou frekvenci MN. Zpětná analýza, využívající data ze stacionárního monitoringu v 15-denních intervalech před odběrem až do 75. dne odhalila vliv koncentace k-PAU naměřené až dva měsíce před vlastním odběrem biologického materiálu na frekvenci MN. Podobný výsledek, vliv expozic znečišťujícím látkám tři měsíce před odběrem, byl již dříve získán s využitím CCA (Sram a kol., 2004a).

Vedle již diskutovaných k-PAU, které měly podle multivariátní analýzy signifikantní vliv na zvýšení frekvence MN, byl studován i vliv VOC, z nichž signifikantní vliv vyšel pro o-xylen. Vliv dalších VOC byl buď na hranici signifikance, nebo nesignifikantní. Zmíněná nižší hladina znečišťujících látek v únoru 2007 se týkala i benzenu, který byl v tomto období na 40% republikového standardu. Podle klasifikace IARC patří benzen mezi lidské karcinogeny a je řazen do první skupiny. Podle některých údajů z literatury nejsou ještě dostatečné důkazy o škodlivém vlivu kontinuálních environmentálních expozic (Duarte-Davidson a kol., 2001), nicméně podle WHO nelze pro benzen doporučit žádnou bezpečnou hladinu (WHO, 2010).

Naše první výsledky byly rovněž porovnávány se studiemi hodnocenými manuálně ve vztahu k věku a kuřáctví účastníků studie. V naší studii nebyl patrný vliv věku na genetické poškození, přestože je to jeden z hlavních faktorů, který zvyšuje frekvenci MN (Bonassi a kol. 2001; Wojda a kol., 2007). Tento zdánlivý rozpor je však dobře vysvětlitelný relativně úzkým věkovým rozpětím účastníků studie. Ve vztahu ke kouření nebyl rozdíl mezi kuřáky a nekuřáky, což je v souladu s publikovanými daty (Bonassi a kol., 2003), která ukazují na zvýšené frekvence MN pouze u silných kuřáků, kteří však nebyli zahrnuti do studované skupiny.

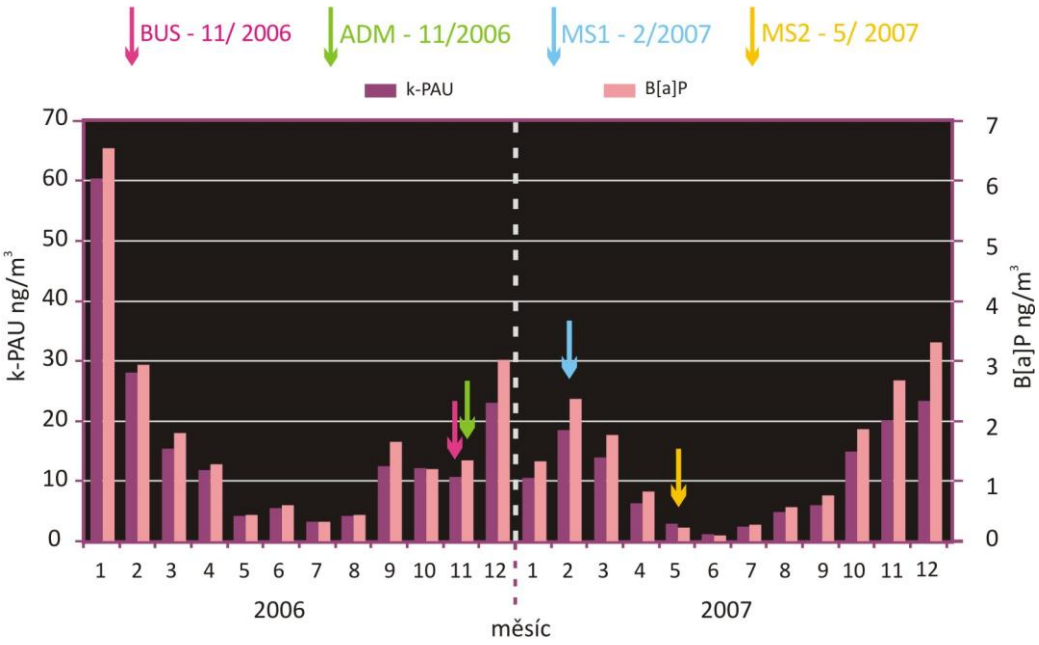
Obecně lze říci, že výsledky této studie ukázaly na možnost využití automatické obrazové analýzy MN v biomonitorovacích studiích. Velkou výhodou pro další studie je rovněž možnost analyzovat rychle vyšší počty DB, a tím získávat přesnější data. Pro další analýzy bylo navrženo hodnocení 2000-3000 DB.

5.2.2. Současný stav aplikace automatické obrazové analýzy MN s využitím systému Metafer od MetaSystems (příloha 7)

V roce 2010 bylo připravováno speciální číslo časopisu Mutagenesis (publikováno v lednu 2011), shrnující veškeré poznatky o aplikaci mikronukleového testu, jehož součástí se stal i příspěvek týkající se současného stavu automatické obrazové analýzy MN s využitím systému Metafer od MetaSystems. Náš příspěvek, stejně tak jako ostatní, byl rozdělen do tří celků: 1. Významné úspěchy, 2. Limitující faktory a 3. Doporučení pro budoucí výzkum. V prvním je kromě historie a metodických aspektů popsán proces automatické analýzy. Hlavní je však souhrn konkrétních aplikací z publikovaných studií, demonstrující použitelnost diskutovaného systému. Dostupné publikace se týkají optimalizace a validace analýzy (Schunck a kol., 2004; Varga a kol., 2004; Maes a kol., 2007), výzkumu ve vztahu k nádorovým onemocněním (Varga a kol., 2005; Varga a kol., 2006), radiační biodosimetrie (Thierens a kol., 2009; Willems a kol., 2010) a biomonitorovacích studií vzniklých v naší laboratoři, které tvoří největší podíl dat (Rossnerova a kol., 2009a; Rössnerová a kol., 2009b; Rössnerová a kol., 2010b; Šrám 2010; Rossnerova a kol., 2011b).

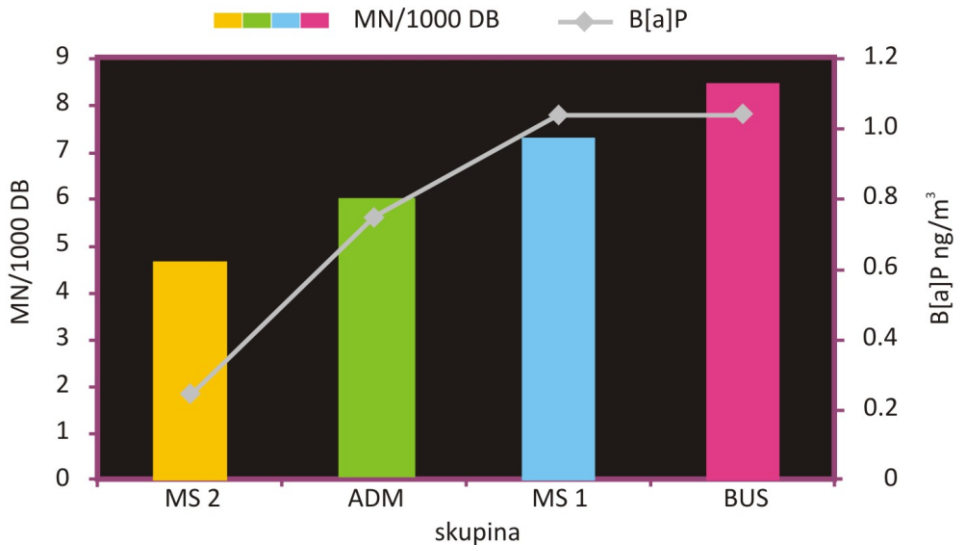
Soubor zde shrnutých našich dat obsahuje výsledky celkem z 529 analýz, které kromě výsledků ve vztahu ke koncentracím B[a]P demonstrují možnosti systému ve vztahu k počtům hodnocených DB. Zatímco v prvních projektech bylo hodnoceno 1000 DB na vzorek, v dalších došlo k rozšíření na 3000 a u skupiny laboratorních pracovníků byla dokonce hodnocena maxima, která se pohybovala kolem 6000 DB na vzorek. Výsledky vlivu B[a]P na skupinu městských strážníků v roce 2007 byly již diskutovány ve vztahu k příloze 6. Další výsledky se týkají skupin řidičů autobusů a administrativních pracovníků sledovaných v listopadu 2006 (Rössnerová a kol., 2009b). Vývoj koncentrací k-PAU a B[a]P ze stacionárního monitoringu v letech 2006 a 2007 v lokalitě Praha-Smíchov je znázorněn na

obrázku 10. Naměřená personální expozice B[a]P byla u řidičů autobusů 1,04±0,76 ng/m^3, což je překvapivě identická hodnota jako u městských strážníků v únoru 2007. Frekvence MN u skupiny řidičů autobusů byla však vyšší (8,48±3,17MN/1000 DB), což je v souladu s vyšším věkem řidičů autobusů (50 let). U zmíněné skupiny administrativních pracovníků bylo zjištěno 5,92±2,82 MN/1000 DB. To odpovídalo nižší personální expozici B[a]P (0,75±0,36 ng/m^3). Kromě těchto výsledků byla porovnávána citlivost metodiky ve vztahu k CCA, která je dlouhodobě osvědčenou metodikou pro hodnocení poškození genetického materiálu u profesionálně exponovaných osob (Rossner a kol., 1995; Sram a kol., 2004a). Naše analýzy ve vztahu ke znečištění ovzduší ukázaly na vyšší citlivost analýzy MN, a to pravděpodobně vzhledem k vyšším počtům analyzovaných buněk, které u CCA představují pouze 100 metafází. Výsledky z dalšího studovaného souboru, ve kterém je hodnoceno genetické poškození u 175 dětí z Ostravy, jsou podrobně komentovány v diskusi k příloze 8. Výsledky 212 automatických obrazových analýz MN z roku 2006 a 2007 u skupin řidičů autobusů, administrativních pracovníků a městských strážníků obecně ukázaly na dostatečnou citlivost metodiky při personálních expozicích B[a]P v rozmezí 0,24 a 1,04 ng/m^3 (obrázek 11). V únoru let 2009 a 2010 byla provedena opakovaná měření u skupiny městských strážníků z Prahy. Naměřené frekvence MN byly v souladu s hodnotou zjištěnou ve stejném období roku 2007. Personální expozice B[a]P pro rok 2009 byla 0,80±0,55 ng/m^3 a pro rok 2010 2,80±1,87 ng/m^3. Výsledky v roce 2010 ukazují na potřebu zaměřit se detailně na vliv koncentrací až 60 dní před odběrem, jak bylo analyzováno dříve (Rossnerova a kol., 2009a), a rovněž na koncentrace dalších znečišťujících látek, jako jsou PM2,5 či benzen, a dále na vliv faktorů životního stylu a porovnání úrovní genové exprese. Naproti tomu je test provedený u osmi laboratorních pracovníků indikativní k zamyšlení nad rychlým efektem po dramatickém zvýšení expozice k-PAU. Bylo provedeno celkem 16 automatických analýz s hodnocením okolo 6000 DB na analýzu. Do pokusu bylo zahrnuto osm pracovníků laboratoře, z nichž každý byl sledován dvakrát. Čtyři strávili v lednu 2010 tři týdny v Ostravě a 4 zůstali po celou dobu v Praze. Personální expozice podle v té době studovaných skupin v Ostravě byly pro k-PAU 99,84±92,96 ng/m^3 a pro B[a]P 14,71±13,31 ng/m^3. Zvýšení frekvence MN u skupiny, která strávila tři týdny v Ostravě, o 62% ukázala na významnou reakci organismu, který není na podobnou zátěž připraven (Šrám 2010).



Obr.10:

Koncentrace k-PAU a B[a]P ze stacionárního monitoringu v letech 2006 a 2007 v lokalitě Praha-Smíchov (Rössnerová a kol., 2009b).



Obr.11:

Frekvence MN a koncentrace B[a]P z personálního monitoringu u skupin sledovaných v letech 2006 a 2007 (vysvětlivky barev viz obrázek 11) (Rössnerová a kol., 2009b).

Závěrečná souhrnná analýza frekvence MN stanovené touto metodou ve vztahu k věku u diskutovaných vzorků ukázala, že data z automatické obrazové analýzy jsou srovnatelná s daty publikovanými v ramci projektu HUMN (Bonassi a kol., 2001).

Jako limitující faktor při srovnání s manuálním hodnocením preparátů barvených Giemsa je některými autory (Fenech, 2010) vnímán fakt, že tato automatická analýza není určená k hodnocení buněk podle nuklearity, viability, či např. k hodnocení NPM, což však nebývá hlavním záměrem většiny studií. Zjištěná frekvence MN může být velmi nepatrně snížena, neboť systém rozpoznává pouze oddělená, nikoliv dotýkající se MN.

Z doporučení pro budoucí aplikaci a výzkum bylo navrženo stejně jako v projektu HUMN maximálně optimalizovat protokol kultivace a zpracování PBL a přípravy mikroskopických preparátů, které má hlavní vliv na optimální nastavení klasifikátorů. Využít barvení DAPI v kombinaci s dalšími fluorescenčními sondami (např. centromerickými, telomerickými či lokus-specifickými) pro získání dalších informací o obsahu a mechanismu formování MN. Dále byla doporučena širší aplikace na různé typy buněčných linií, což bylo zatím publikováno v jediné práci využívající myší buňky (Cariou a kol., 2010). V neposlední řadě je tento způsob analýzy výzvou pro založení nového mezinárodního projektu typu HUMN.

I přes zmíněné limitující faktory byl systém vyhodnocen jako výhodná pomůcka při analýze MN v PBL, a to na základě toho, že posouvá možnosti analýzy na úroveň high-throughput, limituje subjektivitu při hodnocení a podle dostupných studií byl nejvíce prověřen a je i nadále používán v porovnání s alternativním systémem PathFinder^{TM}Cellscan^{TM} (IMSTAR) (Decordier a kol. 2009; Decordier a kol. 2011).

5.2.3. Faktory ovlivňující frekvenci MN u astmatických dětí v Ostravě (příloha 8)

Jak bylo uvedeno v diskuzi k review v příloze 7, věnuje se další práce hodnocení genetického poškození u skupiny 175 dětí (81 astmatických a 94 kontrolních) z Ostravy Radvanic a Bartovic. Studie byla iniciována faktem, že výskyt bronchiálního astmatu u dětí z této lokality, která představuje oblast s nejvyššími koncentracemi k-PAU, podle lékařských záznamů výrazně převyšuje republikový průměr. Prevalence tohoto onemocnění je podle

celostátního průměru 8-10%, zatímco ve studované lokalitě dosahuje více než 30%. Pro srovnání, v dříve studovaných lokalitách Teplice a Prachatice, které reprezentují průmyslovou a zemědělskou oblast, byla prevalence bronchiálního astmatu v roce 2006 8,8% a 5,5% (Dostál a Šrám, 2010).

Odběry biologického materiálu v této studii byly provedeny v listopadu 2008, kdy průměrná hladina B[a]P měřená stacionárním monitoringem byla 11,4±9,8 ng/m^3. Maximální naměřená hodnota v tomto roce byla 92 ng/m^3 a hladina 1 ng/m^3 B[a]P, která představuje hodnotu cílového imisního limitu do roku 2012, byla splněna pouze v červnu, přičemž z celkového počtu 61 měření byla 51 x překročena. U dětí ve věku 6-12 let jsme se kromě vlivu znečištění ovzduší zaměřili na studium vlivu věku, pohlaví, příjmu vitaminů, kouření, genetických polymorfismů a oxidačního poškození na frekvenci mikrojader.

Tato práce byla rovněž provedena s využitím automatické obrazové analýzy mikrojader, která se v předchozích studiích ukázala jako vhodná metodika pro testování genotoxického účinku znečišťujících látek při nízkých personálních expozicích B[a]P (Rossnerova a kol., 2009a; Rossnerova a kol., 2011a). Zvýšená přesnost výsledku byla navíc zajištěna hodnocením 3000 DB/vzorek. Podle předchozích výzkumů jsou děti citlivější ke znečištěnému ovzduší než dospělí, neboť jejich imunitní systém není ještě plně vyvinut (Jedrychowski a kol., 2010). Porovnání genotoxického poškození astmatických a zdravých dětí ukázalo na podobnou expozici znečišťujícím látkám u obou skupin. Tato studie byla v porovnání s předchozími studiemi, kde byla analyzována frekvence MN, provedena v podmínkách s výrazně vyššími expozicemi B[a]P. Získané výsledky naznačují nelineární závislost mezi expozicí a účinkem, což je patrné rovněž z nedávno publikované studie z Číny (Duan a kol., 2009), která porovnává pracovníky ocelárny, jejichž expozice byla 926,9 ng/m^3 B[a]P, s kontrolní skupinou zdravotníků exponovaných 49,1 ng/m^3 B[a]P. I přes signifikantní rozdíl v hladině MN nebylo poškození genetického materiálu zdravotníků, kteří byli exponováni vyšším dávkám v porovnání s naší studií, rovněž tak vysoké. V úrovni genetického poškození hraje pravděpodobně významnou roli míra genové exprese, která se může mezi lokalitami výrazně lišit (Leeuwen a kol., 2008) a může zároveň nastartovat odlišnou účinnost reparačních procesů (Rossner Jr. a kol., 2011a). To se zdá, že neplatí u

osob, které krátkodobě změní prostředí, neboť jejich organismus byl adaptován na odlišné podmínky (Šrám, 2010; Rossnerova a kol., 2011a).

Z hlediska věku odpovídají frekvence MN publikovaným datům získaným při automatické analýze (Rossnerova a kol., 2011a) a jsou v souladu s hodnotami z manuálních analýz (Bonassi a kol., 2001; Neri a kol., 2003; Wojda a kol., 2007). Naše výsledky demonstrují navíc vliv pohlaví na frekvenci MN. V kontrolní skupině jsme pozorovali o 16% vyšší frekvenci u dívek, což se velmi blíží publikovanému údaji, který udává 19% nárůst frekvence MN u žen (Bonassi a kol., 2001).

Ze studovaných faktorů životního stylu jsme analyzovali signifikantně nižší ($p<0,01$) hladinu vitaminu C u skupiny astmatických dětí, což je v souladu s výsledky nedávné metaanalýzy, která uvádí, že nižší hladina vitaminu C v séru je asociována s vyšší pravděpodobností výskytu astmatu (Allen a kol., 2009). Překvapivým zjištěním v této studii bylo kouření 15 dětí, a to i těch, u kterých bylo diagnostikováno astma. Dřívější studie potvrdila vliv kouření na frekvenci MN pouze u silných kuřáků (Bonassi a kol., 2003). Naše výsledky u kontrolní skupiny kouřících dětí jsou v souladu s těmito citovanými závěry, nicméně kombinace kouření a astma bronchiale u osmi dětí ve studii již signifikantní riziko ($p<0,05$) pro zvýšení frekvence MN představovala.

V rámci hodnocení individuální vnímavosti jsme se zaměřili na polymorfismy v metabolických genech *EPHX1, GSTM1, GSTT1*, které byly v předchozí studii spojovány s poškozením chromozomů (Iarmarcovai a kol., 2008), avšak výsledky publikovaných studií jsou v této oblasti velmi nekonzistentí, pravděpodobně vzhledem k nedostatečné velikosti studovaných skupin. Frekvence výskytu jednotlivých variant genů odpovídaly v naší studii očekávaným hodnotám pro kavkazskou populaci. Zjištěná signifikantně nižší hladina mikrojader u *GSTT1* negativní varianty genu byla v souladu s výsledky z publikované rozsáhlejší analýzy provedené u 646 osob (Kirsch-Volders a kol., 2006).

Studium oxidačního poškození v této práci vycházelo z hypotézy, že imunitní reakce organismu při tomto zánětlivém onemocnění zvyšuje oxidační poškození biomolekul, což ovlivní poškození genetického materiálu reprezentovaného zvýšenou frekvencí MN. Podle nedávné studie byla hladina 8-oxodG (nejstudovanějšího markeru oxidačního poškození)

mírně zvýšená u astmatických dětí z průmyslových Teplic (Svecova a kol., 2009). Stejný trend publikovali ve vztahu k markerům oxidačního poškození a astmatu i další autoři (Dut a kol., 2008; Nagai a kol. 2008; Caballero Balanza a kol., 2010; Hasan a kol., 2011). Jiná rozsáhlá studie (N=589) však tyto souvislosti nepotvrzuje (Garcia-Larsen a kol., 2009), stejně jako další práce (Louhelainen a kol., 2008), což odpovídá i našim výsledkům. Nejednotnost v závěrech je pravděpodobně dána rozdílným biologickým materiálem, přičemž kondenzát vydechovaného vzduchu nebo sputum byly navrženy jako vhodnější pro další analýzy u astmatiků. Zvýšená hladina MN byla v této studii asociována se zvýšeným poškozením proteinů, čímž se nepotvrdila hypotéza diskutována v příloze 3 o negativní asociaci mezi oxidačním poškozením proteinů a genetickým poškození.

Tato práce je první biomonitorovací studií, která ukázala zvýšené riziko genetického poškození u kouřících dětí trpících astmatem. Vysoké koncentrace B[a]P v ovzduší neměly pravděpodobně zásadní vliv na hladiny MN, nicméně je potřeba tento výsledek ověřit porovnáním s dětmi z oblasti s nízkou koncentrací B[a]P.

5.2.4. Frekvence MN u matek a jejich novorozenců v lokalitách s různým typem znečištění ovzduší (příloha 9)

Další práce se věnuje vlivu znečištěného ovzduší na genetické poškození vyjádřené frekvencí mikrojader u skupin novorozenců a jejich matek. Studie navazuje na předchozí analýzy provedené v zimním období 2007/2008 na těchto modelových skupinách v Praze s využitím metody FISH (příloha 2), kdy jsme se zaměřili především na studium stabilních chromozomálních aberací. V navazující studii ze stejného období 2008/2009 byl analyzován vliv expozice B[a]P, PM2,5 a benzenu na skupinu celkem 178 matek a jejich novorozenců z Prahy a Českých Budějovic. Obě lokality se výrazně lišily v zastoupení studovaných znečišťujících látek. Tříměsíční průměrné koncentrace B[a]P před porodem byly signifikantně nižší v Praze v porovnání s Českými Budějovicemi: 1,9±0,5 ng/m^3 vs. 3,2±0,2 ng/m^3 ($p<0,001$), zatímco situace ohledně PM2,5 a benzenu vykazovala opačný trend (pro PM2,5: 27,0±2,5 µg/m^3 vs. 24,5±0,7 µg/m^3 ($p<0,001$) a pro benzen: 2,5±0,5 µg/m^3 vs. 2,1±0,8 µg/m^3 ($p<0,001$)). Frekvence MN/1000 DB byly 8,35±3,06 vs. 6,47±2,35 ($p<0,001$) pro matky z Prahy

vs. z Českých Budějovic a 2,17±1,32 vs. 3,82±2,43 ($p<0,001$) pro novorozence z Prahy vs. z Českých Budějovic. Skupina matek, které porodily v Českých Budějovicích, byla navíc rozdělena na dvě podskupiny podle trvalého bydliště ve městě, nebo v okolních vesnicích.

Výsledky získané u novorozenců byly v souladu s naším očekáváním a odpovídaly již dříve pozorovaným vlivům B[a]P na chromozomální aberace (Sram kol.2007b; Rossnerova a kol., 2009a; Rossnerova a kol., 2011a). Stejný trend výsledků byl rovněž zjištěn při porovnání novorozenců z města a ze zemědělské oblasti v Mexiku (Levario-Carrillo a kol., 2005). Mírně vyšší hodnoty pro novorozence i matky z vesnic v porovnání s městem pro Českobudějovicko ukazují pravděpodobně na rozdílné způsoby vytápění v uvedených oblastech. Porovnání genetického poškození matek z Prahy a Českých Budějovic však vykazovalo v porovnání s výsledky u novorozenců opačný trend. Podobný trend výsledku byl nedávno publikován v dánské studii, kde novorozenci z oblasti s intenzivní dopravou měli signifikantně vyšší frekvenci MN, než novorozenci z oblasti se slabým dopravním zatížením, na rozdíl od matek, kde byl trend opačný (Pedersen a kol., 2009). Vyšší hladina PM2,5 a relativně nízká, nicméně dlouhodobě vyšší koncentrace benzenu může být vysvětlením výsledku zjištěného u matek z Prahy. Podobný vliv zvýšených koncentrací PM2,5 na chromozomální aberace hodnocené metodou FISH byl již pozorován v předchozí studii (Rossner Jr. a kol., 2011b). Podle evropské studie je dlouhodobá expozice PM spojována s významnými zdravotními riziky (Pelucchi a kol., 2009). Zmíněné koncentrace benzenu jsou diskutovány ve vztahu k průniku placentou. Je známo, že vysoké koncentrace benzenu placentou pronikají a podílejí se na formování chromozomálních aberací. Situace ve vztahu k nízkým koncentracím, jako byla i naše, však není zatím dostatečně prostudována (IARC, 1987). Z našich výsledků vyplývá, že matky byly ovlivněny expozicí benzenu, na rozdíl od novorozenců, kde lze předpokládat protektivní účinek placenty při naměřených nízkých dávkách. Tento závěr byl podpořen výsledky z multivariátní analýzy, kde byl zároveň zahrnut faktor věku matek, kouření a vzdělání.

Průměrné hodnoty MN/1000 DB pro celé studované skupiny 178 matek a 178 novorozenců a vzájemné signifikantní rozdíly mezi těmito skupinami byly ve vztahu k věku v souladu s publikovanými daty (Bonassi a kol., 2001; Pedersen a kol. 2009; Rossnerova a kol, 2011a). Analýza vlivu věku matek a otců na frekvence MN novorozenců neukázala signifikantní rozdíl. V naší minulé studii (Rossnerova a kol., 2010a) bylo pětinásobné zvýšení

genetického poškození u dětí starších matek spojováno s frekvencí stabilních chromozomálních aberací. Hodnoty získané pro acentrické fragmenty, které reprezentují nestabilní chromozomální aberace, jsou v souladu s výsledky získanými u mikrojader v této studii, ale i s dalšími publikovanými daty (Lope a kol., 2010).

V naší studii byl rovněž hodnocen vliv kouření na frekvence MN. V souladu s publikovanými daty nebyl pozorován vliv na genetické poškození u skupiny matek (Bonassi a kol., 2003) na rozdíl od novorozenců, jejichž matky byly na základě laboratorní analýzy identifikovány jako kuřačky, což je rovněž v souladu s literaturou (Zalacain a kol., 2006). Signifikantně nižší porodní váha těchto novorozenců, která byla v naší studii nižší o 7,4% ($p<0,05$), odpovídala publikovaným datům (Dejmek a kol., 2002; Anderka a kol., 2010).

V této studii jsme se podrobně zabývali i dalšími faktory, jako byl BMI matek před těhotenstvím, délka těhotenství, druh porodu, vzdělání matek a příjem vitaminů A a E. Hodnoty BMI naznačovaly souvislosti s frekvencí MN u matek, kdy nejvyšší hodnoty byly získány u žen s podváhou a klesaly nesignifikantně s nárůstem hmotnosti. Podobný trend byl rovněž pozorován v BioMadridské studii (Lope a kol., 2010).

Celkový závěr z této studie po analýze všech zmíněných faktorů ukazuje na rozdílnou citlivost studovaných skupin k rozdílným hladinám studovaných znečišťujících látek. Frekvence MN u novorozenců byla především ovlivněna koncentrací B[a]P a kouřením matek na rozdíl od matek, u kterých byl zjištěn vliv benzenu a jejich věku, který byl v rozpětí 18-49 let. Faktor věku rodičů nesouvisel s nárůstem nestabilních chromozomálních aberací.

5.3. Význam cytogenetických metodik pro hodnocení rizika expozice karcinogenům

V našich biomonitorovacích studiích byly postupně aplikovány tři druhy cytogenetických analýz. Nejstarší metodikou je klasická CCA, která je využívána především k hodnocení nestabilních chromozomálních aberací, jako jsou chromozomové a chromatidové zlomy, dále FISH jako relativně nová metodika s širokou aplikací v mnoha dalších oborech pro hodnocení zejména stabilních chromozomálních aberací a alternativní klasická metodika analýzy MN v novém automatickém pojetí. Každá z uvedených metodik má své přednosti, ale i omezení.

Hlavním faktorem pro výběr správné analýzy je kromě její ceny a pracnosti především interpretovatelnost získaných výsledků.

Srovnávané metodiky, tak jak byly prováděny, mají různou možnost kontroly kvality hodnocení aberantních buněk. Nefluorescenčně barvené preparáty pro CCA mohou být opakovaně hodnoceny a dlouhodobě skladovány, nicméně fotodokumentace hodnocených aberantních buněk není většinou systematicky prováděna. Fluorescenčně barvené preparáty pro analýzu FISH mají vzhledem ke slábnoucímu signálu fluorescenčních sond omezenější dobu použití, nicméně jejich hodnocení je spojeno s kompletní dokumentací všech aberantních buněk, které jsou archivovány. Preparáty barvené DAPI pro automatickou obrazovou analýzu MN jsou z hlediska dlouhodobějšího použití zatíženy stejným problémem jako preparáty pro FISH. Získané galerie DB jsou kompletním záznamem všech analyzovaných buněk a mohou být dlouhodobě skladovány pro pozdější diskuzi. Mikroskopické preparáty pro všechny zmíněné analýzy mohou být odbarvovány a opakovaně znovu barveny.

Jak již bylo uvedeno, je CCA osvědčenou a bohatě prověřenou metodikou pro hodnocení poškození genetického materiálu u profesionálně exponovaných osob (Rossner a kol., 1995; Sram a kol., 2004a). Při analýzách je hodnoceno 100 metafází (u skupin nad 20 osob), popř. 200-300 (pro menší skupiny či individuální hodnocení). Pro interpretaci expozice skupinových analýz jsou pak důležité následující hranice frekvencí aberantních buněk: 0-2% AB.B. svědčí o biologicky neefektivní expozici genotoxickým látkám, která je lidským organismem pravděpodobně tolerována, 2-4% AB.B. ukazuje na zvýšenou, organismem již netolerovanou expozici a 4 a více % AB.B. vypovídá o vysoké expozici (Rössner a kol., 2000). Porovnání citlivosti CCA s metodou FISH pro hodnocení vlivu environmentálních expozic k-PAU u skupin městských strážníků ukázalo v našich studiích na nižší citlivost CCA (Sram a kol., 2007a; Sram a kol., 2007b), která byla pravděpodobně dána typem hodnocených aberací. Podobné porovnání bylo provedeno ve vztahu k automatické obrazové analýze MN u skupin řidičů autobusů a administrativních pracovníků. Zde byla jako citlivější vyhodnocena analýza MN, která je spojena s hodnocením minimálně 1000 DB (Rössnerová a kol., 2009b).

Zmíněná zjištění vedla k preferenci metodik FISH a MN v našich dalších studiích. Obě tyto metodiky jsou ve srovnání s CCA výrazně nákladnější. FISH kromě vstupních nákladů pak především cenou fluorescenčních sond a analýza MN jakožto jinak levná metodika je

ve svém automatickém pojetí zatížena výraznými vstupními náklady. Z hlediska rychlosti hodnocení zaujímá nesporné prvenství automatická obrazová analýza MN, při které je připravena galerie nasnímaných DB s předtipovanými objekty s MN již během 3-5 minut (podle hustoty objektů). Nespornou výhodou je rovněž možnost kontinuálního a bez dozoru prováděného scanování osmi a při aplikaci přídavného systému až osmdesáti preparátů. Automatická analýza navíc umožňuje jednoduše zvýšit počty analyzovaných buněk, což je u manuálních metodik spojeno s další výraznou časovou ztrátou a při hodnocení velkých skupin je tedy prakticky nemožné.

Celková interpretace výsledků biomonitorovacích studií ve vztahu k použité cytogenetické metodě (FISH popř. MN) naznačuje podobné trendy pro skupiny z Prahy, i pro hodnocené skupiny z Ostravy (podrobněji rozebráno v kapitole *5.4.*). Podobnost v citlivosti metodik byla demonstrována na pokusu provedeném u 4 laboratorních pracovníků cestujících do Ostravy (exponovaní) a 4 osob zůstávajících v Praze (kontrola) (Šrám 2010; Rossnerova a kol., 2011a). Pokus provedený v lednu 2010 byl spojen s vysokými počty hodnocených buněk (průměry na osobu: 5116 metafází pro FISH a 5926 DB pro analýzu MN). Obě metodiky ukázaly na výrazný nárůst chromozomálních aberací u exponované skupiny, která strávila 3 týdny na Ostravsku, zatímco poškození u kontrolní skupiny se ani u jedné z aplikovaných metodik téměř nezměnilo.

I přes celkovou interpretační podobnost výsledků získaných metodou FISH a MN v souboru těchto studií byly pozorovány i některé dílčí rozdíly, a to zejména u městských strážníků studovaných v roce 2007 (Rossnerova a kol., 2009a; Rossner Jr. a kol., 2011b), které pravděpodobně souvisí s různou citlivostí metodik k dalším znečišťujícím látkám, jako je PM2,5, a rovněž s typem studovaných aberací. Pro aplikace v dalších studiích je jistě dostupnější analýza MN (nejfrekventovaněji používaná ve světě), a to bez ohledu na to, zda laboratoř má či nemá možnost automatické analýzy. Naproti tomu je analýza FISH unikátní možností analyzovat stabilní chromozomální aberace, které jsou z hlediska možných zdravotních rizik podstatně závažnější. Pro aplikaci v dalších studiích se nám jeví jako optimální souběžné využití obou diskutovaných metodik, které se svou výpovědní hodnotou vzájemně doplňují.

5.4. Interpretace výsledků vlivu znečištěného ovzduší na frekvenci chromozomálních aberací

Hlavním posláním našeho výzkumu byl monitoring vlivu znečištěného ovzduší na zdravotní stav obyvatel České republiky s využitím řady biomarkerů. Nedílnou součástí tohoto hodnocení se stal i cytogenetický výzkum. Tato práce rekapituluje postupně výsledky jednotlivých studií, které byly získané převážně s využitím metodik FISH (přílohy 1-5) a automatické obrazové analýzy MN (přílohy 6-9). V této podkapitole bych se chtěla pokusit o hledání souvislostí mezi studiemi a jejich celkovou interpretací.

Prezentované studie byly prováděny převážně v Praze, ale také v Českých Budějovicích a Ostravě. Studie provedené výhradně v Praze jsou komentovány v přílohách 1-4 a 6 a hodnotí výsledky získané na modelové skupině městských strážníků, řidičů autobusů, administrativních pracovníků, pracovníků garáží, matek a jejich novorozenců. Další lokalita, České Budějovice, je ve vztahu k matkám a novorozencům porovnávána s Prahou v příloze 9. První výsledky vztahující se k Ostravě se objevují v příloze 7, kde byl zároveň publikován přehled výsledků z automatických obrazových analýz vztahujících se k Praze. Další studie, která již cíleně porovnává výsledky mezi Ostravou a Prahou, je v příloze 5. Výhradně na Ostravu je zaměřená studie věnující se astmatickým dětem v příloze 8.

Výsledky získávané v našich prvních studiích v Praze převážně na modelových skupinách městských strážníků ukazovaly na vhodnost používaných biomarkerů pro hodnocení rizika expozice studovaným znečišťujícím látkám, zejména B[a]P. Z cytogenetických studií to bylo v celé řadě prací (Beskid a kol., 2007; Sram a kol., 2007a; Sram a kol., 2007b; Rossnerova a kol., 2009a; Sram a kol., 2011), ale tyto trendy byly zjištěny i ve vztahu k dalším biomarkerům jako jsou adukty DNA (Binkova a kol., 2007; Topinka a kol., 2007; Sram a kol., 2011), či oxidační poškození (Rossner Jr. a kol., 2007; Rossner Jr. a kol., 2008a; Rossner Jr a kol., 2008b). Automatická obrazová analýza MN ukázala navíc v Praze dostatečnou citlivost při velmi nízkých personálních expozicích B[a]P v rozmezí 0,24-1,04 ng/m^3 (přílohy 6-7). Výsledky cytogenetického poškození však byly ovlivňovány i dalšími znečišťujícími látkami, jako jsou PM2,5 a benzen (přílohy 3, 6, 9), jejichž výkyvy v koncentracích měly rovněž vliv na získané výsledky.

Ve srovnání s pražskými výsledky byly hodnoty jednotlivých biomarkerů v Ostravě, v níž koncentrace k-PAU výrazně přesahují nejen hodnoty v Praze, ale i v dalších oblastech Evropské unie, překvapivě nízké. Výsledky byly rozebrány a komentovány v přílohách 5 a 8. Kromě již publikovaných dat je na základě našich dalších výsledků z analýzy genové exprese prokázáno, že expresní profily se mezi lokalitami výrazně liší (Líbalová a kol., 2010, nepublikovaná data). Podle statistických analýz, které provedl Dr. Hans Gmuender (Genedata AG, Basel), byl tento trend patrný jak mezi lokalitami Ostrava a Prachatice v projektu zaměřeném na astmatické a zdravé děti, tak mezi Ostravou a Prahou v dalším projektu, který hodnotil především skupiny městských strážníků a administrativních pracovníků. Příklad konkrétního rozdílu v expresi reparačních genů byl komentován v příloze 5.

Zcela unikátní na poli těchto výsledků se stala malá, zato široce diskutovaná skupina 4 osob, která změnila prostředí a pražské podmínky vyměnila na 3 týdny za ostravské, na rozdíl od kontrolní skupiny kolegů, kteří zůstali po celou dobu ve stejném pražském prostředí. Výrazný nárůst chromozomálních aberací analyzovaný oběma z cytogenetických metodik je mostem k propojení závěrů z obou lokalit. Organismus adaptovaný na pražské ovzduší při tak výrazné změně prostředí zareagoval velmi intenzivně v porovnání s osobami, které žijí v prostředí s výrazně vyšší zátěží, avšak jsou na něj adaptovány třeba již zmíněným odlišným nastavením genové exprese. Tuto hypotézu potvrdila nejnovější statistická analýza Dr. Hanse Gmuendera, která ukázal na nezměněnou genovou expresi u osob, které strávily tři týdny na Ostravsku.

Obdobný příklad paralelní k rozsahu našich studii na lidské populaci je ve světě těžko vystopovatelný, což podtrhuje unikátnost tohoto výzkumu jako celku. V příloze 8 byl již diskutován vliv vysokých koncentrací B[a]P v Číně (Duan a kol., 2009). Studium osob v různě znečištěných oblastech je celkem dobře proveditelné, nicméně pro další výzkum je velmi obtížné studovat větší skupiny ve vztahu k výrazným změnám životního prostředí, zejména pokud nás zajímá konkrétní lokalita.

Naše závěry se nijak nevylučují s výroky o škodlivosti znečištěného ovzduší, která je na Ostravsku jasně ukázána zvýšenou prevalencí astmatu u dětí (Dostál a Šrám, 2010), či významně zvýšenou celkovou standardizovanou úmrtností i úmrtností způsobenou

zhoubnými nádory a respiračními chorobami v porovnání s Českou republikou (Skorkovský a kol., 2010). Zároveň přetrvává potřeba dalšího studia osob z Ostravska, aby byly jasně definovány reakce biomarkerů, případně reakce na měnící se podmínky.

6. Závěr

Hlavní závěry práce, které zároveň naplňují stanovené cíle, jsou následující:

1. Vliv nízkých koncentrací k-PAU byl dobře patrný jak u skupin hodnocených metodou FISH, tak automatickou obrazovou analýzou MN. Výsledky ukazují jak na význam sezonní variability v koncentracích B[a]P (přílohy 1, 4, 6, 7), tak i na nesporný vliv dalších znečišťujících látek, jako jsou PM2,5 a benzen (přílohy 3 a 9).

2. Poměr zastoupení jednotlivých druhů aberací se s věkem signifikantně mění (příloha 2). Významný je zejména nárůst stabilních translokací, oproti pozvolnějšímu nárůstu nestabilních acentrických fragmentů. Vyšší věk matky může negativně ovlivnit frekvenci stabilních chromozomálních aberací novorozence.

3. Byla zavedena automatická obrazová analýza mikrojader jako vhodná metodika pro aplikaci v biomonitorovacích studiích. Naše práce (příloha 6) se zároveň stala první publikovanou biomonitorovací studií provedenou s využitím tohoto typu analýzy. Vhodnost a věrohodnost hodnocení byla potvrzena v dalších studiích (přílohy 7-9), kde byly navíc ve vztahu k věku, pohlaví a kouření publikovány srovnatelné závěry, jako prezentuje HUMN projekt z výsledků manuální analýzy.

4. Výsledky získané s využitím automatické obrazové analýzy ukázaly na vysokou citlivost metodiky zejména pro hodnocení vlivu nízkých koncentrací k-PAU. Byl identifikován dvouměsíční expoziční interval, který měl vliv na formování MN. Provedené analýzy rovněž přinášejí nové poznatky o vlivu vysokých koncentrací k-PAU, kde hraje pravděpodobně významnou roli odlišná úroveň genové exprese (přílohy 5 a 8).

5. Kromě vlivu konkrétního typu znečištění ve studované lokalitě byl v našich jednotlivých studiích ve vztahu k frekvenci chromozomálních aberací demonstrován rovněž vliv věku, pohlaví, negativní vliv kouření u dětí s bronchiálním astmatem a kouření matek na novorozence, vzdělání v souvislosti se stravovacími návyky a rovněž byl diskutován vliv vitaminů a konkrétních genetických polymorfismů.

6. Obě z aplikovaných cytogenetických metodik mají své nesporné výhody. Metoda FISH je unikátní možností hodnotit stabilní chromozomální aberace, zatímco analýza MN, jako ve světě nejpoužívanější cytogenetická metoda vyniká v kombinaci s automatickým hodnocením především svou rychlostí. Přesto, že trendy výsledků obou metodik byly většinou vzájemně srovnatelné, doporučujeme pro další aplikace souběžné využití metody FISH i analýzy MN, které se svou výpovědní hodnotou mohou vzájemně doplňovat.

7. Výsledky této práce (přílohy 1 a 6) společně s dalšími studiemi se staly podklady pro závěry WHO (WHO, 2010), které ukazují na nebezpečí expozice B[a]P > 1 ng/m^3 pro indukci poškození DNA.

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8. Seznam zkratek

%AB.B.	procento aberantních buněk	percentage of aberrant cells (%AB.C.)
15-F2t-IsoP	15-F2t-isoprostan	15-F2t-isoprostane
8-oxodG	8-oxo-7,8-dihydro-2‘-deoxyguanosin	8-oxo-7,8-dihydro-2‘-deoxyguanosine
APEX1	apurinová/apirimidinová endonukleáza	apurinic/apyrimidinic endonuclease
ADH1B	alkoholdehydrogenáza-2	alcohol dehydrogenase-2
ALDH2	acetaldehyd dehydrogenáza-2	acetaldehyde dehydrogenase-2
B[a]P	benzo[a]pyren	benzo[a]pyrene
BER	bázová excizní reparace	base excision repair
BMI	index tělesné hmotnosti	body mass index
BPDE	benzo[a]pyren diolepoxid	benzo[a]pyrene diolepoxide
BRCA1	protein asociovaný se zvýšenou citlivostí k nádoru prsu typu 1	breast cancer type 1 susceptibility protein
CCA	konvenční cytogenetická analýza	conventional cytogenetic analysis
CYP1A1	cytochrom P4501A1	cytochrome P4501A1
CYP2E1	cytochrom P4502E1	cytochrome P4502E1
DAPI	4´-6-diamidino-2-fenylindol	4´-6-diamidino-2-phenylindole
DB	dvoujaderné buňky	binucleated cells (BNC)
EPHX1	epoxidhydroláza 1	epoxide hydrolase 1
ERCC2 (XPD)	protein zúčastněný v nukleotidové excizní reparaci	excision repair cross-complementing rodent repair deficiency; protein involved in nucleotide excision repair
ETS	environmentální tabákový kouř	environmental tobacco smoke
F_G/100	genomická frekvence translokací na 100 buněk	genomic frequency of translocation per 100 cells
FISH	fluorescenční in situ hybridizace	fluorescent in situ hybridization
GSTM1	glutation S-transferáza M1	glutatione-S-transferase M1

GSTP1	glutation S-transferáza P1	glutatione-S-transferasa P1
GSTT1	glutation S-transferáza T1	glutatione-S-transferase T1
HPRT	Hypoxantin-guanin fosforibosyltransferáza	hypoxanthine-guanine phosphoribosyltransferase
HUMN projekt	Projekt lidská mikrojádra; Mezinárodní projekt pro hodnocení frekvence mikrojader v lidské populaci	Human MicroNucleus project; The International Collaborative Project on Micronucleus Frequency in Human Populations
$HUMN_{XL}$ projekt	Mezinárodní projekt pro hodnocení frekvence mikrojader v buňkách lidské bukální sliznice	The International Collaborative Project on the Micronucleus Frequency in Human Exfoliated Buccal Cells
IARC	Mezinárodní agentura pro výzkum rakoviny	International Agency for Research on Cancer
k-PAU	karcinogenní polycyklické aromatické uhlovodíky	carcinogenic polycyclic aromatic hydrocarbons (c-PAHs)
LIG4	ligáza 4	ligase 4
MN	mikrojádra	micronuclei
MRE11	reparační protein pro opravu dvouřetězcových zlomů	double-strand break repair protein
MRN	proteinový komplex složený z MRE11, RAD50 a NSB1	protein complex consisting of MRE11, RAD50 and NBS1
MTHFR	methylentetrahydrofolát reduktáza	methylene tetrahydrofolate reductase
MTR	5-methyltetrahydrofolát-homocystein methyltransferáza	5-methyltetrahydrofolate-homocysteine methyltransferase
NBS1	protein nibrin	protein nibrin
NHEJ	nehomologní rekombinace	non-homologous end-joining
NPM	nukleoplasmatický můstek	nucleoplasmic bridge (NPB)
OECD	Organizace pro hospodářskou spolupráci a rozvoj	Organisation for Economic Co-operation and Development
OGG1	8-oxoguanin DNA glykosyláza	8-oxoguanine DNA glycosylase
p21	protein p21	protein p21
p53	protein p53	protein p53

PAINT protokol	Protokol pro identifikaci a klasifikaci chromozomálních aberací	Protocol for Aberration Identification and Nomenclature
PM2,5	prachové částice o aerodynamickém průměru < 2,5 µm	particulate matter of aerometric diameter < 2.5 µm
PBL	lidské periferní lymfocyty	peripheral blood lymphocytes
qPCR	kvantitativní PCR	quantitative PCR
RAD50	DNA reparační protein	DNA repair protein
ROS	reaktivní formy kyslíku	reactive oxygen species
SCE	výměna sesterských chromatid	sister chromatid exchange
SOD	superoxiddismutáza	superoxide dismutase
VAPS	Univerzální vzorkovač znečištění ovzduší	Versatile Air Pollution Sampler
VOC	Volatilní organické látky	volatile organic compounds
WHO	Světová zdravotnická organizace	World Health Organization
XPD (ERCC2)	xeroderma pigmentosum D; protein zůčastněný v nukleotidové excizní reparaci	xeroderma pigmentosum D; protein involved in nucleotide excision repair
XRCC (1, 4, 5, 6)	DNA reparační proteiny	X-ray repair complementing defective repair in Chinese hamster cells (1, 4, 5, 6); DNA repair proteins

9. Přílohy 1-9

Příloha 1:

R.J. Sram, O. Beskid, A. Rossnerova, P. Rossner, Z. Lnenickova, A. Milcova, I. Solansky, B. Binkova

Environmental exposure to carcinogenic polycyclic aromatic hydrocarbons – the interpretation of cytogenetic analysis by FISH

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Environmental exposure to carcinogenic polycyclic aromatic hydrocarbons—The interpretation of cytogenetic analysis by FISH

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Abstract

The capital city of Prague is one of the most polluted localities of the Czech Republic. The effect of exposure to carcinogenic polycyclic aromatic hydrocarbons (c-PAHs) adsorbed onto respirable air particles (<2.5 μm) on chromosomal aberrations was studied in a group of city policemen (street patrol, aged 34 ± 8 years) working in the downtown area of Prague and spending daily >8 h outdoors ($N = 61$) in months of January and March 2004. Ambient air particles (PM10, PM2.5) and c-PAHs were monitored using Versatile Air Pollution Sampler (VAPS), and personal exposure was evaluated using personal samplers during working shift. Chromosomal aberrations were analyzed by fluorescent in situ hybridization (FISH) and conventional cytogenetic analysis. Urinary cotinine, plasma levels of vitamins A, E and C, folate, total cholesterol, HDL, LDL cholesterols and triglycerides were also analyzed as possible effect modifiers. During the sampling period the particulate air pollution monitored by VAPS was in January versus March as follows: PM10 55.6 μg/m^3 versus 36.4 μg/m^3, PM2.5 44.4 μg/m^3 versus 24.8 μg/m^3, c-PAHs 19.7 ng/m^3 versus 3.6 ng/m^3, and B[a]P 4.3 ng/m^3 versus 0.8 ng/m^3. Significant differences were observed for all FISH endpoints studied for the sampling in January and March (%AB.C. = 0.27 ± 0.18 versus 0.16 ± 0.17, $p < 0.001$, $F_G/100 = 1.32 \pm 1.07$ versus 0.85 ± 0.95, $p < 0.01$, AB/1000 (aberrations/1000 cells) = 4.27 ± 3.09 versus 2.59 ± 2.79, $p < 0.001$) while conventional cytogenetic analysis did not reveal any differences in the frequency of chromosomal aberrations. Factors associated with an increased level of translocations by FISH indicated the effect of age, cholesterol, LDL-cholesterol and vitamin C. We may conclude that FISH indicates that the city policemen in Prague represent a group of increased genotoxic risk. This is the first study reporting that translocations induced by c-PAHs in peripheral lymphocytes last only several weeks.

Keywords: Chromosomal aberrations; Conventional cytogenetic analysis; Fluorescence in situ hybridization; Whole chromosome painting; Environmental exposure to carcinogenic polycyclic aromatic hydrocarbons

Abbreviations: c-PAHs, carcinogenic polycyclic aromatic hydrocarbons; B[a]P, benzo[a]pyrene; PM10, particulate matter < 10 μm; PM2.5, particulate matter < 2.5 μm; CCA, conventional cytogenetic analysis; FISH, fluorescence in situ hybridization; PBL, peripheral blood lymphocytes; %AB.C., percentage of aberrant cells

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1. Introduction

Cytogenetic analysis of peripheral blood lymphocytes (PBL) has been accepted as a technique suitable for the biological monitoring of genetic damage in somatic cells since the early 1970s. Up to the present time, it has remained the only suitable assay for biological monitoring of the genetic damage induced in somatic cells by excessive exposure to clastogenic agents in the workplace (Natarajan and Obe, 1980; Carrano and Natarajan, 1988; Albertini et al., 2000; Sram et al., 2004b). Today, chromosomal aberrations in human PBL are recognized as an integrated marker of clastogenic exposure and cancer susceptibility, and are the only biomarker of effect which has been internationally standardized and validated (Hagmar et al., 2004; Bonassi et al., 2005; Rössner et al., 2005; Norppa et al., 2006; Boffetta et al., 2007).

When the whole chromosome painting by fluorescence in situ hybridization (FISH) was introduced in the 1990s in the field of ionizing radiation research (Tucker et al., 1993), the classic cytogenetic analysis of chromosomal damage was supplanted by FISH (Tucker, 2001). In comparison with conventional cytogenetic analyses (CCA), which detect particularly unstable types of aberrations, FISH using whole chromosome painting was developed as a rapid and sensitive method of detecting structural rearrangements, especially reciprocal translocations (Stronati et al., 2001; Duran et al., 2002). The FISH technique detects translocations, which are long lasting injuries likely transferred through many cell cycles. These types of chromosomal changes may circulate in PBL for a long period of time and may be related to cancer.

While the FISH technique is highly suitable for analyzing a low-dose radiation exposure (Hsieh et al., 2001; Awa, 2003), available data are still scarce as to ability to detect effect of exposures to chemical carcinogens. However, the FISH painting technique appears to be more sensitive than the conventional technique to detect the genomic frequency of translocations induced by various chemical agents (Verdorfer et al., 2001); e.g. for occupational exposure to acrylonitrile, 1,3-butadiene, ethylbenzene (Sram et al., 2004a) and environmental exposure to carcinogenic polycyclic aromatic hydrocarbons (c-PAHs) (Sram et al., 2007).

Atmospheric pollution by c-PAHs from incomplete combustion represents a relevant environmental hazard and has been associated with a considerable amount of adverse health effects in humans. This prompted a study on the ability of ambient air particulate pollution in Prague to induce chromosomal aberrations in city policemen and "unexposed" controls by two methods: conventional method and FISH. This same cohort of subjects was evaluated during the winter and found that ambient air pollution to c-PAHs during this period significantly increased the genomic frequency of translocations in the city policemen compared to controls, which was not observed by CCA (Sram et al., 2007). In this current study, we followed the same city policemen who were evaluated repeatedly in January and March, when the significant difference between the exposure to c-PAHs was observed.

2. Materials and methods

2.1. Subjects and sampling

The study population consisted of 61 city policemen (ambient exposure during the street patrol), nonsmokers working in the Prague downtown and spending daily >8 h outdoors. The questionnaires on personal medical history and life-style (smoking, alcohol consumption, eating habits) had to be filled in by all participants. In addition, analysis of cotinine level in urine and vitamins A, C, E, and folate, as well as lipids in plasma were done to obtain objective status for smoking and diet.

All participating subjects were healthy volunteers, who signed an informed consent form and could cancel their participation at any time during the study according to Helsinki II declaration. Any person with medical treatment, radiography or vaccination up to 3 months before sampling was not included in the study.

The blood and urine samples were collected at the end of working shift, by venipuncture into vacutainers containing sodium heparin, coded and transported to the laboratory. The urine samples were kept at −80 °C until cotinine analysis. Blood samples were processed within 24 h.

The sampling of this group was carried out in January and March 2004 when the highest air pollution levels were expected. Personal exposure monitoring using personal samplers for collection of $PM_{2.5}$ particles from ambient air was performed during whole working shift as previously described (Binkova et al., 1998). Quantitative chemical analysis of carcinogenic PAHs (c-PAHs), including benz[a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[ghi]perylene, benzo[a]pyrene, chrysene, dibenz[ah]-anthracene and indeno[cd]pyrene, was performed by HPLC with fluorimetric detection according to the EPA method in the laboratories of the certified company ALS Czech Republic, Prague (certification: EN ISO CSN IEC 17025). The personal monitoring was supplemented with daily air pollution data from two monitoring stations located in the downtown and suburban areas. During the sampling period the particulate air pollution monitored by VAPS was in January versus March as follows: PM10 55.6 versus 36.4 $\mu g/m^3$, PM2.5 44.4 versus 24.8 $\mu g/m^3$, c-PAHs 19.7 versus 3.6 ng/m^3, and B[a]P 4.3 versus 0.8 ng/m^3.

2.2. Lymphocyte cultures for cytogenetic analysis

Cultures of peripheral blood lymphocytes from whole venous blood were established within 24 h after the collection. The cultures were prepared in RPMI 1640 medium (Sevapharma, Prague, Czech Republic) supplemented with 20% calf serum (Biochrom AG, Berlin, FRG) and 1% Phytohaemaglutinin (Murex, UK). Two identical cultures were set up from each sample, cultivated at 37 °C, and harvested after 48 h for further conventional staining with Giemsa solution (all cells being in the first division) and after 72 h (to obtain a sufficient number of metaphases) for FISH painting. Two hours before the end of cultivation, colchicine (Fluka, Buchs SG, Switzerland) was added at the final concentration of 1.25×10^{-6} M. The cells were collected by centrifugation, resuspended in a hypotonic 0.075 M KCl solution, and fixed in acetic acid/methanol according to a standard procedure (Carrano and Natarajan, 1988; Rössner et al., 2002).

2.3. FISH analysis

The cell suspensions were stored at −20 °C until painting by FISH. One day before painting, the cell suspensions were dropped on cold and wet slides and left to dry at room temperature overnight. FISH analysis using commercial whole chromosome painting (WCP) probes differing in colors (Cambio, UK) for chromosomes #1 (biotinylated) and #4 (FITC-labeled) was carried out according to the manufacturer's chromosome painting protocol (Rubes et al., 1998). Probes were placed on slides, sealed with rubber cement, and incubated at 37 °C in a moisture chamber overnight. After washing and detecting the signal from chromosome #1 with Streptavidin-Cy3, the slides were mounted in antifade solution Vectashield (Vector Laboratories, Burlingame, CA) with DAPI to counterstain unpainted chromosomes. The slides from each culture were coded and stored at 4 °C in the dark until analysis. One thousand of metaphases were examined for each subject under a fluorescent microscope equipped with a triple-band pass filter for visualization of DAPI (blue), FITC (green) and Cy3 (red) signals.

Aberrant cells were classified according to the Protocol for Aberration Identification and Nomenclature (PAINT) (Tucker et al., 1995) and recorded by ISIS 4.4.16 software (MetaSystem GmbH, Germany) as translocations (t), reciprocal translocations (rcp), dicentric chromosomes (dic), acentric fragments (ace), and insertions (ins). Other analyzed parameters were the percentage of aberrant cells (%AB.C.), aberrations per 1000 cells (AB/1000) and the number of color junctions (NCJ). Genomic frequencies (F_G) of stable chromosomal exchanges were calculated according to Lucas and Sachs (1993) using the equation: $F_G = F_{rg}/2.05\,[f_r(1-f_r)+f_g(1-f_g)-f_tf_g]$. F_{rg} is the translocation frequency measured by FISH after two-color painting, f_r and f_g are the fractions of the genome painted red and green, respectively.

2.4. Conventional cytogenetic analysis

The cell suspension from last fixation step was dropped onto the slides, and the slides were air-dried and stained with 5% Giemsa solution (pH 6.8). The slides from each culture were coded. One hundred well-spread metaphases with 46 ± 1 centromeres were examined per donor. The following four categories of chromosomal aberrations were evaluated: chromatid and chromosome breaks, and chromatid and chromosome exchanges. Gaps were recorded, but they were not scored as aberrations. Cells bearing breaks or exchanges were marked as aberrant cells (AB.C.) (Carrano and Natarajan, 1988).

2.5. QA/QC

Cytogenetic analysis by FISH with whole chromosome painting and the conventional cytogenetic analysis were carried out according to the Standard operating procedures (SOPs) issued for the Czech Republic (Rossner, 2003). Both methods are highly reproducible. Variability is given by the stochastic distribution of cells on slides and the low frequency of analyzed changes to the total number of analyzed cells (FISH approximately $1{:}10^3$, conventional cytogenetic analysis $1{:}10^2$).

2.6. Determination of vitamins

Vitamin C (ascorbic acid) was determined by HPLC in plasma (Kiyoh and Megumi, 1993).

Vitamin E (alpha-tocopherol) and vitamin A were determined by HPLC with UV-detection after *n*-heptane extraction (Driskell et al., 1982).

The CEDIA folate kit (Roche Diagnostics) was used for the analysis of folates in plasma. Adsorption was measured on the ELISA Reader Spectra (TECAN) at wavelength 415 nm (with the reference wavelength 630 nm and the limit of detection 0.6 ng/ml). According to Roche Diagnostics, the level of folate in healthy population corresponds to 2.7–16.1 ng/ml (6.1–36.5 nmol/l).

2.7. Plasma lipids

The plasma levels of triglycerides, total cholesterol, LDL and HDL cholesterol were determined spectrophotometrically using Sigma diagnostic kits and appropriate standards.

2.8. Cotinine analysis

Urinary cotinine levels as a marker of active and passive smoking were analyzed by radioimmunoassay (Langone and van Vunakis, 1982). Subjects with cotinine levels greater than 500 ng/mg of creatinine were considered active smokers.

2.9. Statistical analysis

Three kinds of c-PAHs were used in the statistical analysis—individual 48 h c-PAHs personal monitoring, envi-

ronmental c-PAH monitoring 48 h in the time of sampling, and 10 or 30 days means of environmental c-PAH in periods up to 60 days preceding to sampling for evaluate real impact of c-PAHs. Data from personal questionnaire about type of heating, type of commuting, partner's smoking was tested for possible c-PAH sources.

The univariate statistics for all studied variables were calculated. To evaluate the associations between cytogenetic endpoints of both staining methods and the potential modifying factors of air pollution, vitamins, cotinine, and plasma lipids, we calculated the bivariate correlations or used the analysis of variance (ANOVA). Mann–Whitney rank sum *U*-test for comparison of two samples, Kruskal–Wallis ANOVA by ranks and Spearman rank correlation test for evaluation of relationship between chromosomal aberrations and other variables were used.

For evaluation comprehensive models with all confounding factors together, we used *STATISTICA ©Multiple Linear Regression* direct on continual dependent or independent variable values and similar models on three level scales split by variables distribution tertils.

The *STATISTICA General Classification and Regression Trees* module (*GC&RT*) analyzing facility we used to build classification and regression trees for predicting continuous dependent variables by regression and estimate importance of influence to dependent variables (Breiman et al., 1984).

3. Results

Based on the personal monitoring data the policemen were during working shift exposed in January versus March to significantly higher concentrations of c-PAHs 9.07 ± 9.94 versus 3.46 ± 4.65 ng/m^3 ($p < 0.001$), as well as B[a]P 1.58 ± 1.39 versus 0.39 ± 0.64 ng/m^3 ($p < 0.001$), respectively (Table 1). All other characteristics as vitamins, lipids, and cotinine levels did not differ between the both sampling periods.

Table 1
Characteristics of city policemens ($N = 61$, mean ± S.D.)

	January 2004	March 2004
Age (years)	34 ± 8	34 ± 8
Benzo[a]pyrene (ng/m^3)	1.58 ± 1.39***	0.39 ± 0.64
c-PAHs (ng/m^3)	9.07 ± 9.94***	3.46 ± 4.65
Vitamin A (μmol/l)	3.63 ± 1.01	4.15 ± 0.97
Vitamin C (μmol/l)	64.06 ± 15.54	59.85 ± 12.28
Vitamin E (μmol/l)	24.77 ± 8.25	30.27 ± 8.76
Folate (μmol/l)	28.73 ± 22.35	31.98 ± 19.33
Cholesterol (mmol/l)	4.06 ± 1.24	4.14 ± 1.11
HDL-cholesterol (mmol/l)	1.04 ± 0.30	1.15 ± 0.33
LDL-cholesterol (mmol/l)	2.30 ± 0.83	2.54 ± 0.79
Triglycerides (mmol/l)	1.43 ± 1.05	1.38 ± 0.87
Cotinine (ng/mg creatinine)	19 ± 19	17 ± 13

*** $p < 0.001$.

Concentrations of c-PAHs and B[a]P detected by personal monitoring during 48 h according to multiple linear regression are significantly correlated with stationary monitoring in this period (c-PAHs: $R = 0.20$, $p < 0.05$ and B[a]P: $R = 0.15$, $p < 0.05$) and to commuting by car ($R = 10.04$, $p < 0.01$ and $R = 1.54$, $p < 0.01$, respectively) by data from personal questionnaire (Table 2).

FISH analysis detected significant difference of chromosomal aberrations between the sampling in January and March in all parameters determined: (%AB.C. $= 0.27 \pm 0.18$ versus 0.16 ± 0.17, $p < 0.001$, $F_G/100 = 1.32 \pm 1.07$ versus 0.85 ± 0.95, $p < 0.01$, AB/1000 (aberrations/1000 cells) $= 4.27 \pm 3.09$ versus 2.59 ± 2.79, $p < 0.001$, Table 3).

By conventional cytogenetic analysis, no differences were observed in the frequency of AB.C. between January and March in the nonsmoking policemen (Table 3). Comparing %AB.C. between the FISH and CCA methods in the sampling in January, a high correlation was observed ($R = 0.3472$, $p < 0.01$), this effect was not observed in the sampling in March.

As chromosomal aberrations detected by both cytogenetic methods correspond to exposure during several weeks/months, we also calculated for each policemen the average exposure to c-PAHs and B[a]P 10–60 days before the sampling. The best model for multiple linear regression fitted to the 10 days mean, 40–50 days before sampling FISH data (%AB.C.: $R = 1.85$, $p < 0.05$; $F_G/100$: $R = 0.47$, $p < 0.05$; AB/1000: $R = 0.07$, $p < 0.05$). No such a relationship was observed for the conventional cytogenetic analysis.

Table 2
Possible additional c-PAHs sources according to questionnaires

Risk	*N*	%
Policeman—ex-smoker	12	20.0
His partner—smoker	10	16.7
Traffic load in residence area		
None	19	31.7
Low	12	20.0
Medium	22	36.7
High	7	11.7
Heating		
Gas	7	11.7
Coal	2	3.3
Wood	2	3.3
Fireplace	1	1.7
Commuting		
By car	7	11.7
By bus	29	48.3
By bicycle	3	5.0
On foot	9	15.0

Table 3
Cytogenetic analysis—FISH and CCA results (means ± S.D.)

	FISH				CCA	
	N	%AB.C.	F_G/100	AB/1000	*N*	%AB.C.
January 2004	60	0.27 ± 0.18***	1.32 ± 1.07**	4.27 ± 3.09	61	2.07 ± 1.48
March 2004	61	0.16 ± 0.17	0.85 ± 0.95	2.59 ± 2.74	61	1.84 ± 1.28

(Mann–Whitney test).
*** $p < 0.001$.
** $p < 0.01$.

Using linear regression for FISH, genomic frequency of translocations F_G/100 was significantly affected by age, cholesterol, LDL-cholesterol and vitamin C (Table 4). The frequency of AB.C. (%AB.C.) and AB/1000 was significantly affected only by age. The frequency of chromosomal aberrations by conventional method was affected only by HDL-cholesterol (regression coefficient $R = 1.388$, $p < 0.05$).

Using classification and regression trees for predicting continuous dependent variables by regression, we estimated the importance impact to FISH (Fig. 1) and to conventional cytogenetic analysis (Fig. 2). %AB.C. by FISH was significantly affected by age, LDL-cholesterol, vitamin C, and folate. %AB.C. by CCA was significantly affected by c-PAHs, HDL-cholesterol, cotinine, vitamin C, and folate.

4. Discussion

The frequency of chromosomal aberrations, detected mostly as translocations, by FISH with a whole chromosome painting for chromosomes #1 and #4, was significantly higher in January than in March and corresponded to the differences between the exposure to c-PAHs.

The original idea about the stability of translocations in PBL induced by ionizing radiation (Lucas and Sachs, 1993; Lucas, 1997, 1999) developed during the last period due to whole chromosome painting technique. Some authors believe that frequency of translocations in stable cells (i.e., those not containing unstable aberrations in any chromosome) is persistent over time (Tawn and Whitehouse, 2003; Lindholm and Edwards, 2004). However, other studies indicate that translocations lifetime is more complicated than originally thought. In rat experiments, Tucker et al. (1997) observed the decline of translocations already 4 days after the exposure to 1 and 2 Gy, respectively. Gardner and Tucker (2002) put forward an idea why translocation frequencies decline over time: translocations may be eliminated as a by-product of selection against dicentrics, or a fraction of translocations may themselves be cell-lethal, i.e. one or both of the DNA double-strand breaks that led to the translocation may have occurred within a gene or regulatory sequence that is essential for cell growth.

Tucker et al. (2005a,b) studied the persistence of translocations following in vitro irradiation of peripheral blood lymphocytes of two donors by 0.2–4.0 Gy. With the doses as low as 0.2–0.3 Gy the frequency of translocations decreased within 7 days. This suggests an unknown efficiency with which radiation-induced DNA damage is either recognized or repaired. It may be therefore expected that translocations induced by any dose of radiation could reasonably be anticipated to diminish in frequency over time (Tucker et al., 2005b).

Originally, we speculated that a long-term exposure to ambient air concentrations of c-PAHs 20–25 ng/m^3,

Table 4
Factors affecting genomic frequency of translocations (F_G/100) by FISH, *R* (significance)

	January 2004	March 2004
Age (years)	0.065 ($p < 0.001$)	0.048 ($p < 0.001$)
Benzo[a]pyrene (ng/m^3)	0.045	0.170
c-PAHs (ng/m^3)	0.005	0.040
Cholesterol (mmol/l)	0.223 ($p = 0.059$)	0.166
HDL-cholesterol (mmol/l)	0.738	0.256
LDL-cholesterol (mmol/l)	0.310 ($p = 0.083$)	0.258
Vitamin C (μmol/l)	−0.017 ($p < 0.078$)	−0.009

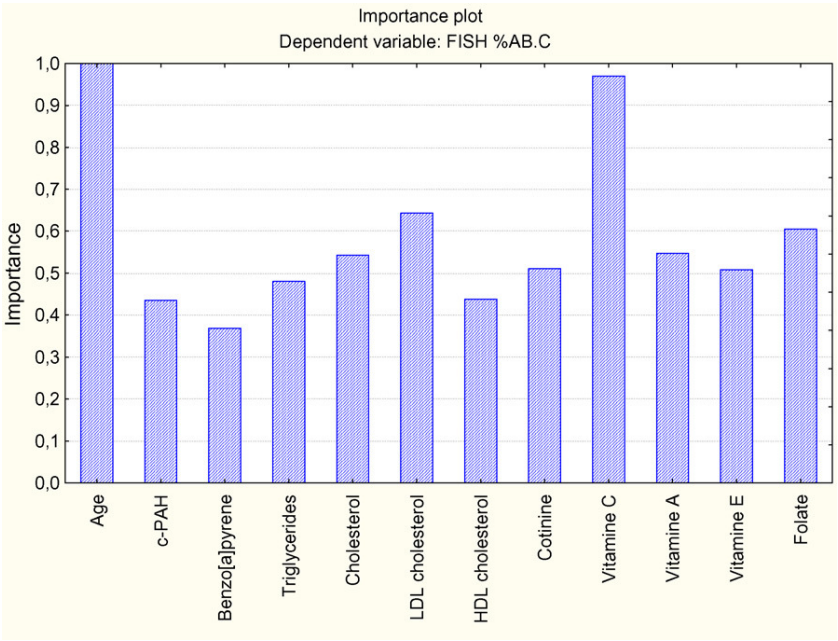

Fig. 1. The importance impact of dependence variables to FISH cytogenetic analysis (%AB.C.).

B[a]P 2.9–3.5 ng/m^3 (Binkova et al., 2003) may already damage DNA, inducing DNA adducts which remain unrepaired and are finally manifested as structural chromosomal aberrations—translocations. Our results are first ones indicating that translocations induced by c-PAHs in human PBL survive only several weeks. They indicate that the life expectancy of translocations induced by chemical carcinogens may correspond to the selection against translocations induced by ionizing radiation, as was observed, e.g. for mice (Spruill et al., 2000;

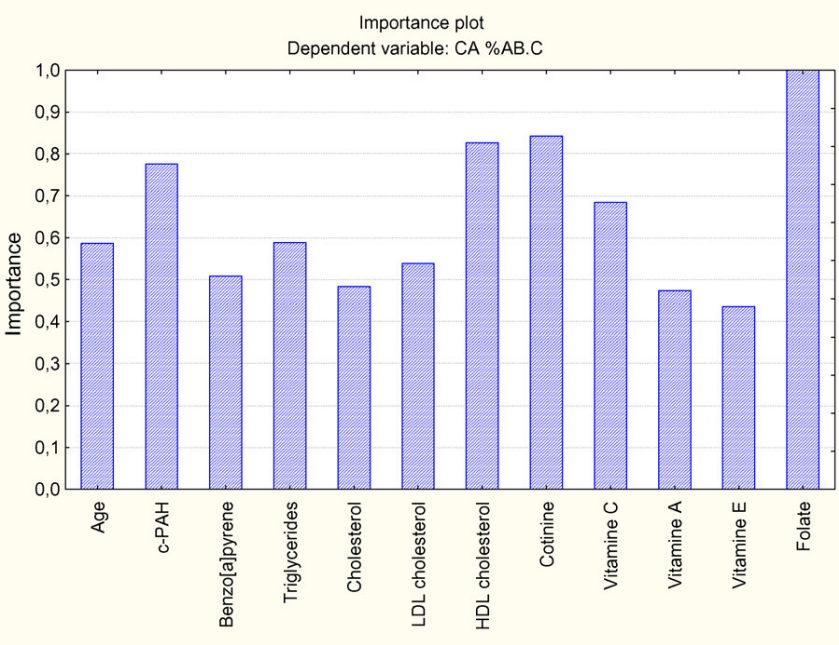

Fig. 2. The importance impact of dependence variables to conventional cytogenetic analysis (%AB.C.).

Tucker et al., 2004) as well as astronauts (George et al., 2005).

We previously observed a similar situation with a group of workers exposed to ethyl benzene, where we found decreased genomic frequency of translocations several months after a significant decrease of exposure (Sram et al., 2004a).

Conventional cytogenetic analysis detects chromatid and chromosome breaks and exchanges, with the frequency of aberrant cells (%AB.C.) corresponding to the exposure to chemical carcinogens during the previous 3 months (Sram et al., 2004b). Until now the preference for the FISH method was based on its ability to detect stable translocations that reflect exposure over several years (Kleinerman et al., 2006). This idea is particularly important for radiation exposure, where translocations may be induced already in stem cells (Edwards et al., 2005).

This FISH methodology has significant impact for public health. We propose that the results of genomic frequency of translocations detected by FISH can be used in the same way as results of CCA to evaluate the risk of occupational exposure to carcinogens, and ultimately improve working conditions. This advanced cytogenetic analysis is widely used in the Czech Republic due to the fact that the Hygiene Service has accepted the theoretical assumption that the frequency of aberrant lymphocytes is a surrogate indicator for the genetic damage caused by clastogens in target tissues. Cytogenetic analysis of chromosomal aberrations has been proposed as a useful tool for checking whether a safe occupational maximum allowable concentration (MAC) has truly been established (Sram and Kuleshov, 1980; Sram, 1981). The MAC of a chemical is defined as the maximum concentration of a gas, vapor or aerosol in the working atmosphere that, according to contemporary scientific knowledge, did not adversely affect the health of exposed humans (Sram et al., 2004b).

Previously we observed the ambient air concentrations of c-PAHs in the city center of Prague during winter induced simultaneously DNA adducts and structural chromosomal changes by FISH (Binkova et al., 2007; Sram et al., 2007). As DNA adducts indicate an increased risk of atherosclerosis (De Flora et al., 1997; Binkova et al., 2002), it was postulated that city policemen in downtown of Prague are at higher risk of cardiovascular diseases. The increased levels of chromosome translocations were further related to age, life style factors such as smoking, lipid metabolism, vitamin intake (specifically folate). The effect of occupation in city policemen, detected as chromosomal aberrations, was further modulated by genetic polymorphisms (Sram et al., 2007). Similar results have been observed in the present study with an analogous exposure to c-PAHs, specifically for sampling in January.

Chromosomal aberrations are a biomarker indicating an increased risk for health consequences. Therefore, we may hypothesize that exposure to chemical carcinogens increasing genomic frequency of translocations represents a significant health risk.

Carcinogenic PAHs are adsorbed on fine respiratory particles (PM2.5) and probably represent the most important biologically active group of pollutants (Binkova et al., 2003). As c-PAHs were not originally listed among chemicals, which concentrations should be monitored, stationary monitoring of c-PAHs started in Europe in some countries only recently. It is planned that the immission limit for B[a]P concentration in an ambient air should be 1 ng/m^3/year. Our data indicate that such concentration can still induce genetic injury. Certainly the concentration of chemical carcinogen able to induce translocations should be considered as deleterious.

Chromosomal aberrations detected as translocations by FISH are a sensitive biomarker of effect and can help to evaluate the risk of occupational and environmental exposure to mutagens and carcinogens.

FISH results show a new knowledge about the risk of c-PAHs in polluted air, which may significantly affect human health. This new knowledge should be used for risk assessment, and to determine new standards for long-term environmental c-PAHs exposure.

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Příloha 2:

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Frequency of chromosomal aberrations in Prague mothers and their newborns

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Frequency of chromosomal aberrations in Prague mothers and their newborns

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ABSTRACT

The capital city of Prague is one of the most polluted areas of the Czech Republic. The impact of air pollution on the level of chromosomal aberrations was systematically studied: analyses were performed using fluorescence *in situ* hybridization (FISH) with whole-chromosome painting for chromosomes #1 and #4. In the present study, we analyzed the levels of stable (one-way and two-way translocations) and unstable (acentric fragments) chromosomal aberrations in 42 mothers living in Prague and in their newborns. The average age of the mothers was 29 years (range, 20–40 years). Blood samples were collected from October 2007 to February 2008. The average levels of carcinogenic polycyclic aromatic hydrocarbons (c-PAHs) and benzo[*a*]pyrene (B[*a*]P) in respirable particles (PM2.5), as determined by stationary monitoring, were 21.0 ± 12.3 ng/m^3 and 2.9 ± 1.8 ng/m^3, respectively. We did not observe any effect of either c-PAH or B[*a*]P exposure on the genomic frequency of translocations (per 100 cells, F_G/100) in either group due to their similar exposure during the winter months. The mean values of F_G/100 representing stable aberrations were 0.09 ± 0.13 *vs* 0.80 ± 0.79 ($p < 0.001$) for newborns *vs* mothers, indicating a significant increase of F_G/100 with age. On the other hand, the frequency of unstable aberrations did not differ between the two groups. Our results demonstrate how the patterns of different types of aberration differed between newborns and mothers: we observed 64.3% unstable aberrations and 35.7% stable aberrations in newborns *vs* 19.7% and 80.3% in mothers, respectively. Our results indicate that after birth the frequencies of aberrations are very low and that the aberrations are represented mainly by acentric fragments. The changes observed in mothers show a shift to stable aberrations represented mainly by two-way translocations. The mother's age affected the level of aberrations in newborns: the group of children born to older mothers (31–40 years) had significantly increased F_G/100 levels.

1. Introduction

Carcinogenic polycyclic aromatic hydrocarbons (c-PAHs) are formed as a result of the incomplete combustion of organic matter. They are ubiquitous in the environment and occur mainly in traffic, heating and industrial emissions. c-PAHs are among the most studied environmental pollutants in genetic toxicology. Their deleterious effect on human health has been shown repeatedly [1]. Many factors, such as the density of settlement, traffic, industry, heating routine, airflow, season and/or the surrounding landscape have an impact on the final concentration of pollutants. The higher pollution levels found in larger cities are usually related to traffic of motor vehicles. According to results by Binkova et al. [2] the genotoxicity of c-PAHs adsorbed onto airborne particles poses the most significant health risk from air pollution in the Czech Republic. Environmental exposure to c-PAHs has been associated with an increase in the genomic frequency of translocations in peripheral lymphocytes [3,4]. Recent studies have focused on the most polluted and urbanized places around the world [5–7]. Complex studies, comparing exposure data with individual dietary habits, smoking status, age or susceptibility, are valuable for estimating the risk for DNA damage [8,9].

Chromosomal damage has often been investigated by the use of cytogenetic methods—conventional cytogenetic analysis (CCA), analysis of micronuclei (MN) and fluorescence *in situ* hybridization (FISH). CCA, as a method focused mainly on unstable aberrations such as chromosomal and chromatid breaks, has been used in various studies to investigate the levels of damage in people working in the chemical industry [10]. Pooled European data have shown

Abbreviations: % AB.C., percentage of aberrant cells; AB/1000, aberrations/1000 cells; ace, number of acentric fragments/1000 cells; B[*a*]P, benzo[*a*]pyrene; CCA, conventional cytogenetic analysis; c-PAHs, carcinogenic polycyclic aromatic hydrocarbons; Cy-3, cyanine-3; DAPI, 4′,6-diamidino-2-phenylindole; dic, number of dicentric chromosomes/1000 cells; F_G/100, genomic frequency of translocations/100 cells; FISH, fluorescence *in situ* hybridization; FITC, fluorescein-5-isothiocyanate; ins, number of insertions/1000 cells; MN, micronuclei; NCJ, number of colour junctions/1000 cells; PHA, phytohaemagglutinin; PM2.5, particulate matter < 2.5 μm; rcp, number of reciprocal (two-way) translocations/1000 cells; t, number of all (one-way plus two-way) translocations/1000 cells.

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that chromosomal aberrations are a valuable biomarker of effect [11]. Comparing the impact of environmental air pollution with CCA and FISH, the latter method seems to be more sensitive [4]. Another advantage of the FISH technique is that this method is focused on stable aberrations. Stable translocations, represented mainly by one-way and two-way translocations and insertions, are able to reveal very low levels of genotoxins. This method also shows unstable aberrations represented by acentric fragments, but the results are limited to the painted chromosomes. In addition, the second mitotic cycle will diminish the findings when compared with CCA data, where the first mitotic cycle is usually evaluated [12].

The genomic frequency of translocations (F_G/100) is affected by age as stable aberrations accumulate with time [13]. In a pooled analysis of translocation data in unexposed individuals collected from 16 laboratories around the world (N = 1933) by use of the FISH technique, the effects of age, gender, ethnicity and cigarette smoking on baseline translocation frequency were determined [14]. This comprehensive study also reported results from a group of 296 newborns in which the average F_G/100 was 0.04. Other smaller studies also analyzed the genomic frequencies of translocations in newborns [13,15–17]. Recently, a study was published evaluating the effect of maternal smoking during pregnancy on structural chromosome aberrations in 241 newborns [18].

Our study was initiated by the conflicting data on the levels of chromosomal aberrations in newborns reported by several studies (i.e., low levels of chromosomal aberrations in [13,15] *vs* high levels in [16]). In the present study, we used the FISH technique with whole-chromosome painting for chromosomes #1 and #4 to study the levels of stable and unstable chromosomal aberrations in a group of newborns and their mothers living in Prague, where the exposure to B[*a*]P is several times higher then in New York City [16]. We evaluated the effect of c-PAH exposure on the frequency of chromosomal aberrations in both groups. Our study compares the levels of the genomic frequency of translocations in newborns and their mothers and analyzed the differences in the spectra of stable and unstable aberrations in these groups.

2. Materials and methods

2.1. Study subjects

The study was performed in collaboration with the University Hospital Motol in Prague. The study populations were mothers and their newborns. Mothers, aged 20–40 years, were randomly recruited from the Department of Neonatology, during the period between October 8, 2007 and February 26, 2008. All mothers were non-smokers and spent the time of their pregnancy in the city. Information on the course and outcome of the pregnancy was obtained from medical records. Uncomplicated pregnancies (84% vaginal and 16% Caesarian births, 95% with term deliveries) were included in the study. The average birth weight of newborns was 3395 ± 429 g [median (range): 3530 (2020–4130) g]; 45% of mothers were primiparous. Fifty-five whole venous blood samples from the mothers and 55 cord blood samples from the newborns were collected in sodium-heparinized tubes and stored at 4 °C until processing. Due to the quality of the blood samples, several mothers were excluded from the study. Finally, chromosomal aberrations in 55 newborns and 42 mother–newborn pairs were investigated.

All mothers signed an informed consent form and could cancel their participation at any time during the study, according to the Helsinki II declaration. The ethical committee of the Institute of Experimental Medicine AS CR in Prague approved the study.

2.2. Monitoring of air pollution

Stationary monitoring of particulate matter <2.5 μm (PM2.5), and c-PAHs to assess the level of ambient air pollution and the external exposure of the subjects was performed using a versatile air pollution sampler (VAPS) [19]. The VAPS was located in Prague–Smichov, a city-center area classified according to the European Environmental Register of monitoring stations as a commercial, residential, traffic, and urban location. The daily concentrations of c-PAHs including benzo[*a*]pyrene, benz[*a*]anthracene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*ghi*]perylene, chrysene, dibenz[*ah*]-anthracene, and indeno[*cd*]pyrene were determined after c-PAH extraction from filters and a quantitative chemical analysis performed by HPLC with fluorescence detection according to the EPA method [20].

2.3. Culture of cord and venous blood

Cultures of cord blood and whole venous blood were established within 24 h after blood collection, according to the method described by Rossner et al. [21]. Lymphocyte cultures were set up in tissue-culture flasks, each culture containing 0.6 ml of whole blood and 7.5 ml of medium—cultivation medium for one culture contained 1.06 ml RPMI 1640 (Sevac, Czech Republic), 1.80 ml fetal bovine serum (Biochrom AG, Germany), 4.28 ml distilled water, 2.5 mg glutamine, 0.16 ml $NaHCO_3$ (7.5%, w/v), and 24 μg PHA HA-15 (Biochrom AG, Germany).

Cell cultures for FISH were cultivated at 37 °C and harvested after 72 h of incubation to obtain a sufficient number of mitoses. Colchicine (Fluka) was added to a final concentration of 0.5 μg/ml 2 h before the end of the incubation. The cells were collected by centrifugation, re-suspended in pre-warmed (37 °C) hypotonic solution (0.075 M KCl) for 10 min and fixed in acetic acid/methanol according to the standard protocol [22]. The cell suspensions were stored at −20 °C in fixative. Slides were always prepared using the air-dry method, randomly numbered and, after painting, scored "blind" in numerical order.

2.4. FISH technique

Fresh slides were prepared by dropping a fixed pellet of metaphase cells onto slides, which were stored in ethanol with 1% ether. The protocol to perform FISH with whole-chromosome probes for chromosomes #1 and #4 was adapted from the protocol provided by Cambio (Cambridge, UK) [23]. The counterstaining, following the washes, was with DAPI mixed with Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA) at a final concentration of 0.24 μg/ml.

The analysis of FISH slides was performed using a Metafer version 3.2 automated scanning system from MetaSystems (Altlussheim, Germany) connected to an Axio Imager Z1 microscope (Carl Zeiss, Germany). The microscope was equipped with filters for the visualization of DAPI (blue), FITC (green) and Cy-3 (red) signals. Automatic metaphase detection was performed by the MSearch software module with a 10× objective. AutoCapt software was used in combination with a 63× oil objective for scanning full coloured images at a higher magnification [24]. One thousand metaphases per subject were analyzed. Coloured images with aberrations were collected and analyzed by use of ISIS software version 5.0 (MetaSystems).

All aberrant cells were classified according to the Protocol for Aberration Identification and Nomenclature (PAINT) [25]. The protocol defines translocations as follows: a translocation (t) is a rearranged chromosome with a single centromere and is to be counted as an aberration—the translocated chromosome must exhibit at least two colours; a reciprocal translocation (rcp) is the exchange of genetic material between two chromosomes of different colours; a dicentric chromosome (dic) contains two centromeres from chromosomes of different colours; an acentric fragment (ace) is a linear part of a red or green painted chromosome without a centromere; and an insertion (ins) is acentric chromosomal material inside the chromosome of another colour. All t (one-way plus two-way), rcp (two-way) and ins were calculated as stable aberrations and ace and dic as nonstable aberrations. Insertions were excluded from analysis because none were found in any group. Other analyzed parameters were the percentage of aberrant cells (%AB.C.), aberrations per 1000 cells (AB/1000) and the number of colour junctions (NCJ). All cells with a colour junction or a painted acentric fragment were counted as aberrant cells.

The genomic frequencies (F_G) of stable chromosome exchanges were calculated according to Lucas et al. [26] using the equation: $F_G = F_{rg}/2.05[f_r(1-f_r)+f_g(1-f_g)-f_rf_g]$. F_{rg} is the translocation frequency measured by FISH after two-colour painting, while f_r (red) and f_g (green) are the fractions of the genome [27] (chromosomes #1 and #4 represent 8.28% and 6.39% of the human genome, respectively) painted red and green, respectively.

2.5. Statistical analysis

The data did not follow a normal distribution. Therefore, the non-parametric Mann–Whitney *U* test was used for comparison of the genomic frequency of translocations/100 cells (F_G/100), the percentage of aberrant cells (% AB.C.), the number of colour junctions/1000 cells (NCJ), aberrations/1000 cells (AB/1000), the number of all translocations/1000 cells (t), the number of reciprocal/1000 cells (rcp), the number of acentric fragments/1000 cells (ace), and the number of dicentric chromosomes/1000 cells (dic) between groups. The effect of exposure to environmental pollutants (c-PAHs and B[*a*]P) on F_G/100, %AB.C. and ace was analyzed by binomial logistic regression after transforming the variables into a two-level scale using medians. The analyses were performed with SPSS software (SPSS Inc.-version 17.0).

3. Results

The entire study was conducted during the months in which the levels of c-PAHs are elevated. The average levels of c-PAHs and B[*a*]P determined from stationary monitoring were 21.0 ± 12.3 ng/m^3

Table 1
Concentrations of c-PAHs and B[*a*]P (mean ± SD) measured by stationary monitoring during the collection of samples.

Month	*N*	Prague–Smichov	
		c-PAHs (ng/m^3)	B[*a*]P (ng/m^3)
October 2007	9	14.9 ± 9.1	1.9 ± 1.2
November 2007	10	20.1 ± 13.7	2.7 ± 1.8
December 2007	3	23.3 ± 13.6	3.3 ± 2.0
January 2008	12	17.9 ± 5.1	2.4 ± 0.8
February 2008	8	29.0 ± 20.2	4.4 ± 3.1
All	42	21.0 ± 12.3	2.9 ± 1.8

N: number of mother–newborn pairs.

and 2.9 ± 1.8 ng/m^3, respectively (Table 1). For each mother the exposure to c-PAHs and B[*a*]P at 30 days before delivery was calculated. We did not observe any effect of exposure to above-median levels of c-PAHs on either F_G/100, %AB.C. or ace in either group (Table 2A and B). We also tested for each subject the effect of exposure to c-PAHs on cytogenetic parameters in a 1-month period before delivery, but we did not find any significant association (data not shown). The results obtained for exposure to B[*a*]P were very similar (data not shown).

An overview of the mean values of the measured parameters assessed by FISH in newborns and their mothers is presented in Table 3A and B; the results of the Mann–Whitney test used to compare individual groups are reported in Table 4. The level of the genomic frequency of translocations representing stable aberrations in newborns was generally very low: mean levels ± SD were as follows: F_G/100 = 0.08 ± 0.18 and 0.09 ± 0.13 for all newborns and newborns paired with mothers, respectively. This is underlined by the fact that in 45.2% of the samples no aberrations were found, and 78.6% of the samples were without any translocation. Newborns born to older mothers (age, 31–40 years) had 5-fold higher levels of F_G/100 than newborns born to mothers aged 20–30 years (mean ± SD: 0.15 ± 0.22 and 0.03 ± 0.11, $p < 0.05$ for newborns born to older and younger mothers, respectively) (Table 3A). Although the levels of F_G/100 were higher in older mothers (Table 3B), the differences were not statistically significant (Table 4). Differences in F_G/100 between newborns and mothers were highly significant (mean ± SD: 0.09 ± 0.13 and 0.80 ± 0.79, $p < 0.001$, for newborns and mothers, respectively; Tables 3 and 4). On the other hand, no differences between mothers and newborns were found for unstable aberrations, represented mainly by acentric fragments. The average levels were nearly identical in paired newborns and their mothers (0.43 ± 0.76 *vs* 0.50 ± 1.03, respectively, $p = 0.98$) (Tables 3 and 4).

The frequencies of individual types of chromosomal aberration in newborns and mothers are shown in Fig. 1. Although the number of individual cases with unstable aberrations is almost equal in both groups (acentric fragments: 18 (64.3%) *vs* 21 (18.8%) in newborns *vs* mothers, respectively), this type of aberration was the major

Table 2
Results from logistic regression of c-PAH concentrations affecting F_G/100, %AB.C. and ace in newborns (A) and mothers (B).

A

c-PAHs (ng/m^3)	Newborns		
	F_G/100	% AB.C.	ace
	OR (95% CI), *p*	OR (95% CI), *p*	OR (95% CI), *p*
≤20.1	Reference	Reference	Reference
>20.1	0.67 (0.12–3.86), 0.65	2.10 (0.46–9.62), 0.34	0.79 (0.17–3.72), 0.77

B

c-PAHs (ng/m^3)	Mothers		
	F_G/100	% AB.C.	ace
	OR (95% CI), *p*	OR (95% CI), *p*	OR (95% CI), *p*
≤20.1	Reference	Reference	Reference
>20.1	1.21 (0.28–5.21), 0.80	0.34 (0.06–1.90), 0.22	0.47 (0.08–2.63), 0.39

F_G/100: genomic frequency of translocations/100 cells; % AB.C.: percentage of aberrant cells; ace: number of acentric fragments/1000 cells.

Table 3
Mean ± SD of all parameters assessed by FISH in a group of newborns (A) and mothers (B).

A

Newborns	*N*	F_G/100	%AB.C.	NCJ	AB/1000	t	rcp	ace	dic
All	55	0.08 ± 0.18	0.08 ± 0.10	0.22 ± 0.49	0.89 ± 1.09	0.22 ± 0.49	0.02 ± 0.13	0.40 ± 0.70	n.d.
Paired with mothers	42	0.09 ± 0.13	0.09 ± 0.10	0.24 ± 0.48	0.95 ± 1.13	0.24 ± 0.48	0.02 ± 0.15	0.43 ± 0.76	n.d.
Paired girls	23	0.10 ± 0.20	0.11 ± 0.12	0.26 ± 0.53	1.13 ± 1.30	0.26 ± 0.53	0.04 ± 0.20	0.43 ± 0.71	n.d.
Paired boys	19	0.08 ± 0.15	0.07 ± 0.07	0.21 ± 0.41	0.74 ± 0.85	0.21 ± 0.41	n.d.	0.42 ± 0.82	n.d.
Born to mothers aged 20–30	22	0.03 ± 0.11	0.06 ± 0.09	0.09 ± 0.29	0.64 ± 0.98	0.09 ± 0.29	n.d.	0.45 ± 0.89	n.d.
Born to mothers aged 31–40	20	0.15 ± 0.22	0.13 ± 0.11	0.40 ± 0.58	1.30 ± 1.19	0.40 ± 0.58	0.05 ± 0.22	0.40 ± 0.48	n.d.

B

Mothers	*N*	F_G/100	%AB.C.	NCJ	AB/1000	t	rcp	ace	dic
All paired with newborns	42	0.80 ± 0.79	0.21 ± 0.18	2.21 ± 2.12	3.10 ± 2.93	2.14 ± 2.11	0.86 ± 0.97	0.50 ± 1.03	0.02 ± 0.15
Age 20–30	22	0.76 ± 0.92	0.19 ± 0.92	2.14 ± 2.42	2.68 ± 2.96	2.05 ± 2:46	0.82 ± 1.19	0.36 ± 0.93	0.05 ± 0.21
Age 31–40	20	0.84 ± 0.61	0.23 ± 0.17	2.30 ± 1.73	3.55 ± 2.84	2.25 ± 1.64	0.90 ± 0.62	0.65 ± 1.11	n.d.

N: number of subjects; F_G/100: genomic frequency of translocations/100 cells; % AB.C.: percentage of aberrant cells; NCJ: number of colour junctions/1000 cells; AB/1000: aberrations/1000 cells; t: number of all translocations/1000 cells; rcp: number of reciprocal/1000 cells; ace: number of acentric fragments/1000 cells; dic: number of dicentric chromosomes/1000 cells; n.d.: not detected.

Table 4
Comparison of parameters shown in Tables 1 and 2 by use of the Mann–Whitney test (p).

	F_G/100	% AB.C.	NCJ	AB/1000	t	rcp	ace	dic
Newborns/mothers	<0.001	0.01	<0.001	<0.001	<0.001	<0.001	0.98	0.32
Girls/boys	0.9	0.42	0.9	0.46	0.9	0.36	0.67	1
Mothers' age 20–30/31–40	0.39	0.26	0.46	0.18	0.39	0.21	0.1	0.34
Newborns of mothers 20–30/31–40	<0.05	<0.05	<0.05	<0.05	<0.05	0.29	0.71	1

F_G/100: genomic frequency of translocations/100 cells; % AB.C.: percentage of aberrant cells; NCJ: number of colour junctions/1000 cells; AB/1000: aberrations/1000 cells; t: number of all translocations/1000 cells; rcp: number of reciprocal/1000 cells; ace: number of acentric fragments/1000 cells; dic: number of dicentric chromosomes/1000 cells.

fraction in newborns, considering their low frequency of stable aberrations. The figures also illustrate the ratio between one-way and two-way translocations. One-way translocations were a major fraction in newborns, while two-way translocations prevailed in mothers.

4. Discussion

In the present study we analyzed the effect of c-PAH exposure on the levels of stable and unstable aberrations and compared their levels and spectra in a group of newborns and their mothers. Our results indicate that exposure to above-median levels of c-PAHs was not accompanied by increased frequencies of either type of aberration in either group, probably due to the very narrow range of c-PAH concentrations during the study months. Several studies analyzing the effect of environmental exposures on the levels of stable and unstable aberrations have been published [3,4,16,17,28]. In most of them an effect of environmental pollutants on the levels of aberrations was detected: in city policemen from Prague, Czech Republic, the levels of F_G/100 decreased significantly along with the drop in exposure to c-PAHs in different seasons [4]. Similarly, in police officers from Prague and Sofia, Bulgaria, the levels of stable aberrations were significantly higher than in controls exposed to lower air pollution measured by personal monitors [3]. On the other hand, Pedersen et al. [28] did not find any difference between groups of adults and children residing in two regions of the Czech Republic, probably due to their unusually low exposure to pollutants 1 month before the sampling period. In all these studies the levels of F_G/100 and other parameters were in the range comparable with the results of the present study. Bocskay et al. [16,17] reported a significant association between prenatal exposure to c-PAHs and levels of stable aberrations in 60 newborns of African–American or Dominican origin residing in New York City. Despite substantially lower exposure to environmental pollutants in their study, they observed several-fold higher frequencies of stable aberrations in newborns than we did in the present study. This discrepancy may be explained by the fact that Bocskay et al. did not use the PAINT system for scoring structural aberrations detected by chromosome painting [25] and included deletions among the stable aberrations [16]. Differences in dietary habits between low-income groups of African–American and Dominican women and the mothers in our study may be another reason for the conflicting results. The lack of any significant effect of exposure to air pollutants on the frequency of stable and unstable aberrations in our study may be explained by the relatively narrow range of c-PAH and B[*a*]P concentrations during the study period, as no samples were collected in the spring or summer seasons when the concentrations of air pollutants are lowest.

Our study is unique because it concentrates simultaneously on the levels of stable and unstable aberrations in both newborns and mothers. Apart from the chance to compare the response of a mother and a newborn to environmental pollutants, we also had the opportunity to evaluate the differences in the spectra of stable and unstable aberrations in both age groups. Although studies of cytogenetic damage in newborns are scarce, the data we obtained are comparable with the results of others who used the PAINT system to evaluate their results. The frequency of translocations reported by Pluth et al. [15] (F_G/100 = 0.11) was similar to our observations. The mean translocation frequencies observed in newborns in an international study [14] (F_G/100 = 0.04) were even lower than those found in our study.

The effect of age on the accumulation of cytogenetic damage is well known. It was measured by chromosome painting in the study of Ramsey et al. [13]. In their study, a comparison of the frequencies of stable aberrations, dicentrics, and acentric fragments was performed between newborns and adults aged 19–79 years. The frequencies of stable aberrations in adults *vs* newborns were elevated 10.6-fold, while the increase in unstable aberrations was distinctly less pronounced. In our study, the frequency of stable aberrations in mothers was 9-fold higher than in newborns, while the frequency of acentric fragments remained virtually unchanged. The equal results observed for unstable aberrations in both our groups may be explained by their exposure to the same levels of c-PAHs during gestation. Thus, our results show an interesting change

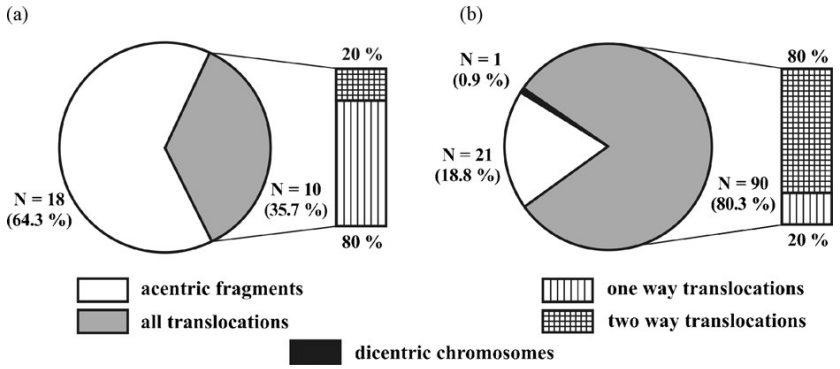

Fig. 1. The pattern of chromosomal aberrations: (a) newborns; (b) mothers; *N*: number of individual types of chromosomal aberration.

in the pattern of chromosomal aberrations with age. Although the number of individual cases with unstable aberrations is almost equal in both groups, this type of aberration is the major fraction in newborns, considering the low frequency of stable aberrations in this group. Age also affects the ratio between one-way and two-way translocations. One-way translocations are a major fraction in newborns, while two-way translocations prevail in mothers. It was reported that the use of whole-chromosome painting without other specific probes (i.e., telomeric probes) may result in higher estimates of the frequencies of one-way translocations and lower frequencies of reciprocal ones [29]. However, in our study the patterns of stable aberrations in both groups were markedly different. Thus, the limitations described above should not change the interpretation of our results. The pattern of chromosomal aberrations in mothers in our study is similar to the pattern found in the control group of 49 subjects in the study by Beskid et al. [30].

It is well known that women conceiving at an older age have a higher frequency of spontaneous abortions and that their fetuses are at a greater risk for serious chromosomal abnormalities. However, our study underlines another important aspect of the risk of conception at older age: the frequency of stable chromosomal aberrations in healthy newborns seems to be affected by the age of the mothers. Thus, newborns born to older mothers, even though seemingly healthy, carry a genome with a higher frequency of defects, which may cause health problems later in life. Also, from the point of view of analyzing the genetic damage resulting from environmental pollution, it appears very important to record the age of the mother when evaluating chromosomal aberrations in newborns, in order to control for this potential confounder.

In conclusion, we did not observe any effect of air pollution on the levels of stable or unstable chromosomal aberrations in either newborns or mothers due to their similar exposure during the winter months. We found differences in the patterns of chromosomal aberrations between newborns and their mothers: different genomic frequencies of translocations, including frequencies of one-way and two-way translocations, and the frequencies of acentric fragments. The levels of stable aberrations in newborns increased with the age of their mothers.

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Příloha 3:

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Oxidative stress and chromosomal aberrations in an environmentally exposed population

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Oxidative stress and chromosomal aberrations in an environmentally exposed population

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ABSTRACT

We investigated the effect of the seasonal variability of environmental air pollutants on oxidative stress and cytogenetic biomarkers in a group of 59 city policemen working in Prague, Czech Republic. The studied group was monitored in February and May 2007. The exposure to environmental pollutants (carcinogenic polycyclic aromatic hydrocarbons, c-PAHs, including benzo[a]pyrene, B[a]P, and particulate matter of aerodynamic diameter < 2.5 μm, PM2.5) was measured by personal and/or stationary monitors. Levels of c-PAHs were significantly higher in winter than spring, while exposure to PM2.5 was higher in May than in February 2007. We did not observe any significant difference between the two seasons for any biomarker of oxidative stress (8-oxo-7,8-dihydro-2′-deoxyguanosine, 8-oxodG, 15-F_{2t}-isoprostane, 15-F_{2t}-IsoP, protein carbonyl levels) or any cytogenetic parameter, including the genomic frequency of translocations (F_G/100), the percentage of aberrant cells (%AB.C.) or the number of acentric fragments (ace). Analyses of associations between oxidative stress biomarkers and cytogenetic parameters showed a negative relationship between protein oxidation and F_G/100, as well as protein oxidation and ace. We further analyzed the effect of air pollution on all subjects regardless of the season. Data from stationary monitors showed that 8-oxodG levels were significantly increased by exposure to PM2.5 over a 2-day period before sampling and by exposure to B[a]P over a 28-day period, days 57–84 before sampling. 15-F_{2t}-IsoP levels were increased after exposure to B[a]P over both 2-day and 3-day periods preceding sample collection and after exposure to c-PAHs over a 2-day period before sampling. %AB.C. was significantly affected by exposure to B[a]P over a 14-day period, days 57–70 before sampling. In summary, our results indicate that the exposure to environmental pollutants affects urinary excretion of 8-oxodG, lipid peroxidation and the frequency of chromosomal aberrations.

1. Introduction

Air pollution is a significant factor negatively affecting the inhabitants of both developed and developing countries. Many studies have dealt with the health effects of polluted air. A recent review indicates that long-term exposure to particulate matter (PM) of aerodynamic diameter < 2.5 μm (PM2.5) increases the risk of nonaccidental mortality by 6% for each 10 μg/m^3 increase in the concentration of pollutants, irrespective of age, gender or geographic location [1]. Exposure to polluted air is also associated with a higher incidence of various diseases, including cardiovascular and pulmonary disorders, as well as cancer [1–10]. Negative effects of exposure to air pollutants are particularly deleterious for children and adolescents, in whom they may affect the development of lung function [3]. PM2.5 consists of respirable particles of dust, soot, liquid and aerosol with various chemicals bound to them. Of many chemicals adsorbed to respirable particles carcinogenic polycyclic aromatic hydrocarbons (c-PAHs) are regarded as one of the most important [11]. c-PAHs are metabolized into reactive intermediates that may bind to DNA and form PAH–DNA adducts, thus causing mutations and increasing cancer risk [12].

There are also other mechanisms underlying the effect of c-PAHs on genetic material. Polycyclic aromatic hydrocarbons (PAHs) may induce oxidative stress when metabolized by aldo-keto reductases to *o*-quinones [13]. These compounds may enter redox cycles and cause oxidative damage to macromolecules by the formation of reactive oxygen species (ROS). Chromosomal aberrations (CA)

Abbreviations: 8-oxodG, 8-oxo-7,8-dihydro-2′-deoxyguanosine; 15-F_{2t}-IsoP, 15-F_{2t}-isoprostane; AB/1000, aberrations/1000 cells; %AB.C., percentage of aberrant cells; ace, number of acentric fragments/1000 cells; B[a]P, benzo[a]pyrene; CA, chromosomal aberrations; c-PAHs, carcinogenic polycyclic aromatic hydrocarbons; Cy-3, cyanine 3; DAPI, 4′,6-diamidino-2-phenylindole; dic, number of dicentric chromosomes/1000 cells; F_G/100, genomic frequency of translocations/100 cells; FISH, fluorescent in situ hybridization; FITC, fluorescein-5-isothiocyanate; GEE, generalized estimating equations; ins, number of insertions/1000 cells; MN, micronuclei; NCJ, number of color junctions/1000 cells; PAH–DNA adducts, polycyclic aromatic hydrocarbon–DNA adducts; PM2.5, particulate matter of aerodynamic diameter < 2.5 μm; rcp, number of reciprocal (two-way) translocations/1000 cells; ROS, reactive oxygen species; t, number of all (one-way plus two-way) translocations/1000 cells.

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are another result of the exposure of cells to PAHs. CA, either unstable (e.g. breaks) or more serious stable translocations, which are conserved in the genome and may lead to changes in the expression of various genes, including oncogenes, may be formed by two different mechanisms. The production of ROS as a result of PAH metabolism and their interaction with DNA may induce strand breaks [14,15]. If double strand breaks are formed and then processed by DNA repair mechanisms (homologous repair or nonhomologous end-joining repair), CA may result [16]. Another pathway leading to CA is initiated by PAH–DNA adducts. The adducts do not form double strand breaks directly, but as byproducts of repair mechanisms [17,18].

Oxidative stress, caused by ROS, is defined as an imbalance between the levels of pro-oxidants and antioxidants. It may arise from endogenous and exogenous sources. While endogenous sources include natural processes, e.g. cellular metabolism or inflammation, exogenous sources arise from the environment, diet or life style. Oxidative stress affects all cellular macromolecules: DNA, lipids, as well as proteins [19]. It changes their function, causing mutations or disseminating oxidative stress to other molecules.

Measuring the levels of molecules modified or induced by oxidative stress has proved to be a useful approach to identify health risks in the environment [20]. Stable CA detected by fluorescence in situ hybridization (FISH) were repeatedly shown to be a sensitive biomarker of exposure to c-PAHs (e.g. [21,22]). The advantage of FISH is its ability to detect the type of CA that are long lasting, transfer through many cell cycles, affect the expression of target genes and thus are directly related to cancer initiation.

In the present study we analyzed the levels of oxidative stress markers (8-oxo-7,8-dihydro-2′-deoxyguanosine, 8-oxodG; 15-F_{2t}-isoprostane, 15-F_{2t}-IsoP, protein carbonyls) and the frequency of chromosomal aberrations in a group of 59 city policemen working in Prague, Czech Republic. We collected the samples in February and May 2007, two seasons that might differ in the levels of air pollution. We investigated the seasonal variability of the biomarkers and their associations with levels of c-PAHs and PM2.5 in the ambient air. Due to the possible effect of ROS on the frequency of CA, we further analyzed the associations between the biomarkers of oxidative stress and CA. We hypothesized that exposure to higher concentrations of ambient air pollutants (PM2.5, c-PAHs) in winter season will be associated with both elevated levels of oxidative stress markers and higher frequency of cytogenetic parameters. We also expected oxidative stress markers to be positively correlated with cytogenetic parameters.

2. Materials and methods

2.1. Subjects and sampling

The study population consisted of 59 city policemen (39 non-smokers, 20 smokers) living and working in the city center of Prague, Czech Republic, spending more than 8 h outdoors. The study subjects were followed in two seasons with supposedly different levels of air pollutants, in February and May 2007. Each participant completed a questionnaire on his personal medical history and life-style. All participants signed an informed consent form and could cancel their participation at any time during the study, according to the Helsinki II declaration. The study was approved by the ethical committee of the Institute of Experimental Medicine AS CR in Prague. Any person who underwent medical treatment, radiography or vaccination up to 3 months before sampling was not included in the study.

Spot urine samples and blood were collected at the end of the working shifts. For each subject, one urine sample and one blood sample was obtained for each season. The blood samples were collected by venipuncture into vacuettes containing sodium heparin. Samples were coded, transported to the Laboratory of Genetic Ecotoxicology and processed within 2 h. Samples to be analyzed for levels of oxidative stress markers were kept in aliquots at −80 °C.

2.2. Exposure assessment

The subjects' exposure to c-PAHs was monitored by personal samplers used by the study subjects during two consecutive days (48 h). For each subject, the personal monitoring was performed once in each season. The samplers were equipped with filters collecting particles of aerometric diameter < 2.5 μm (PM2.5) [23]. After extraction of the filters with dichlormethane, quantitative chemical analysis of c-PAHs (benz[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, dibenzo[a,h]anthracene, benzo[g,h,i]perylene and indeno[1,2,3-cd]pyrene) was performed by HPLC with fluorescence detection according to the EPA method [24] in the certified laboratory ALS Czech Republic, Prague. The extraction of the filters with the organic solvent did not allow us to assess the concentrations of transition metals, other important inducers of oxidative stress.

Ambient air quality during the sampling periods and up to 90 days before sampling was monitored using stationary Versatile Air Pollution Samplers (VAPS) in Prague. The samplers continuously measured the levels of c-PAHs and PM2.5 [11]. The study subjects lived and worked within the range of the samplers.

The concentrations of both pollutants measured before sampling were used to estimate the role of air pollution over various intervals on biomarkers of oxidative stress and cytogenetic parameters. Mean values of concentrations of c-PAHs and PM2.5 in 3-day, 14-day and 28-day intervals up to 3 months before sampling were calculated and compared with the levels of analyzed biomarkers.

2.3. Analysis of oxidative stress markers

2.3.1. 8-oxodG ELISA

Urinary 8-oxodG levels were analyzed by competitive ELISA essentially as previously described [25,26]. Wells were coated with 5 ng of 8-oxoG conjugated with bovine serum albumin (BSA; total volume, 50 μl/well) by drying the plates overnight at 37 °C. Plates were washed with PBS/Tween (0.05% Tween 20 in PBS (phosphate buffered saline)) and blocked with 200 μl/well of blocking buffer (1% FCS in PBS/Tween) for 1 h at 37 °C. After blocking, 50 μl of 8-oxodG standards (concentration range, 1.25–40 ng/ml) and urine samples (diluted 1:1 with PBS) were added followed by 50 μl of primary antibody (JaICA, Japan, clone N45.1, concentration 0.2 μg/ml). After incubation for 1.5 h at 37 °C and washing, 100 μl of secondary antibody conjugated with alkaline phosphatase was added. Another 1.5-h incubation at 37 °C was followed by washing with PBS/Tween and with 0.01% diethanolamine in water. The color was developed by adding 100 μl of *p*-nitrophenyl phosphate substrate (1 mg/ml of 1 mol/L diethanolamine) per well and incubating the plates for 30–60 min at 37 °C. The absorbance was measured with a microplate reader at 405 nm. Any samples with inhibition <20% or >80% were repeatedly analyzed either without dilution or with further dilution, respectively. Each sample was analyzed in triplicate. Urinary 8-oxodG concentration was expressed as nmol 8-oxodG/mmol creatinine. To control for the interassay variability, a control sample was analyzed on every plate and the interassay coefficient of variability was calculated. For the analysis of 8-oxodG, the interassay coefficient of variability was 5.7%.

2.3.2. 15-F_{2t}-IsoP Immunoassay

Urinary 15-F_{2t}-IsoP levels were analyzed using immunoassay kits from Oxford Biomedical Research (Oxford, MI, USA) as described by Rossner et al. [26]. Urine samples were thawed to room temperature and diluted 7× using the dilution buffer provided with the kit. Analysis was done according to the recommendations of the manufacturer. According to the manufacturer, the results obtained with the kit correlate well with GC/MS following solid phase extraction ($R > 0.8$). Each sample was analyzed in duplicate. The 15-F_{2t}-IsoP concentration was divided by creatinine levels and expressed as nmol 15-F_{2t}-IsoP/mmol creatinine.

2.3.3. Protein carbonyl assay

The levels of protein carbonyl groups were assessed in blood plasma using a noncompetitive ELISA, as previously described [27], with some modifications [26,28]. Briefly, the oxidized protein standards were prepared by incubation of BSA (50 mg/ml) with 0.73 M H_2O_2 and 0.42 mM Fe^{2+} for 1 h at 37 °C. The reaction was stopped with 40 μM butylated hydroxytoluene. The carbonyl content of the oxidized BSA standard was measured spectrophotometrically. It was then diluted with native (unoxidized) BSA and PBS to give a final carbonyl content of 2.0 nmol/mg protein and protein concentration of 4 mg/ml. Total protein concentration in the plasma samples was measured using Bicinchoninic Acid kit and the samples were diluted with PBS to a final protein concentration of 4 mg/ml. After the derivatization with 2,4-dinitrophenylhydrazine (DNPH), the plate was coated with 200 μl of sample and incubated overnight at 4 °C in the dark. The plate was washed with PBS/Tween (0.05% Tween 20 in PBS) and blocked with 0.1% BSA in PBS for 1.5 h. After another washing step, biotinylated primary anti-DNP antibody (Molecular Probes, OR, USA; diluted 1:1500 with 0.1% BSA, 0.1% Tween 20 in PBS) was added and the plate was incubated at 37 °C for 1 h. Another washing was followed by adding the streptavidin-biotinylated horseradish peroxidase conjugate (Amersham Biosciences, UK; diluted 1:4000 in 0.1% BSA, 0.1% Tween 20 in PBS) and incubated at room temperature for 1 h. Color was developed by adding the tetramethyl benzidine (TMB) liquid substrate system and the reaction was stopped with H_2SO_4 after 15–25 min incubation in the dark. The absorbance was measured with a microplate reader at 450 nm. Each sample was analyzed in triplicate. Plasma protein carbonyl concentration was expressed as nmol carbonyl/ml plasma. The interassay coefficient of variability was 3.1%

Table 1
Exposure to tobacco smoke and environmental pollutants in the winter and spring seasons.

Variable	February 2007 ($N=59$)		May 2007 ($N=59$)		p
	Mean ± SD	Median (min, max)	Mean ± SD	Median (min, max)	
Cotinine (ng/mg creatinine)	515.2 ± 927.9	15.5 (2.1, 2949.3)	854.3 ± 1547.2	16.5 (2.9, 5940.3)	0.37
B[a]P (ng/m^3)[a]	1.03 ± 0.74	0.69 (0.27, 3.63)	0.22 ± 0.47	0.15 (0.15, 3.77)	<0.001
c-PAHs (ng/m^3)[a]	6.03 ± 4.19	4.21 (1.91, 19.5)	2.14 ± 3.29	1.58 (1.58, 26.7)	<0.001
PM2.5 (μg/m^3)[b,c]	26.1 ± 14.3	19.9 (12.0, 57.3)	28.4 ± 2.81	28.5 (25.7, 34.3)	<0.001
B[a]P (ng/m^3)[b,c]	2.12 ± 1.32	1.93 (0.98, 5.01)	0.18 ± 0.08	0.10 (0.10, 0.27)	<0.001
c-PAHs (ng/m^3)[b,c]	16.7 ± 10.1	14.9 (8.3, 38.8)	2.52 ± 0.58	1.99 (1.99, 3.15)	<0.001

[a] Data from personal monitors.
[b] Data from stationary monitors.
[c] Concentrations of PM2.5 measured by stationary monitors showed an opposite trend than levels of B[a]P and c-PAHs.

2.4. Fluorescence in situ hybridization

2.4.1. Whole venous blood cultures

Whole venous blood cultures were established within 24 h after blood collection, according to the method described by Rossner et al. [29]. Cell cultures were cultivated at 37 °C, harvested after 72 h of incubation and processed as described by Rossnerova et al. [30]. The cell suspensions were stored at −20 °C. Slides were prepared using the air-dry method, randomly numbered and after painting scored "blind" in numerical order.

2.4.2. FISH technique

The protocol used to perform FISH with whole chromosome probes for chromosomes #1 and #4 was described in detail by Rossnerova et al. [30]. The analysis of FISH slides was performed using a Metafer version 3.2 automated scanning system from MetaSystems (Altlussheim, Germany) connected to an Axio Imager Z1 microscope (Carl Zeiss, Germany). The microscope was equipped with filters for the visualization of DAPI (blue), FITC (green) and Cy-3 (red) signals. Automatic metaphase detection was performed by the MSearch software module using a 10× objective. AutoCapt software was used in combination with a 63× oil objective for scanning full colored images at a higher magnification [31]. One thousand metaphases per subject were analyzed. Colored images with aberrations were collected and analyzed using ISIS software version 5.0 (MetaSystems).

All aberrant cells were classified according to the Protocol for Aberration Identification and Nomenclature (PAINT) [32]. The protocol defines translocations as follows: a translocation (t) is a rearranged chromosome with a single centromere and is to be counted as an aberration – the translocated chromosome must exhibit at least two colors; a reciprocal translocation (rcp) is the exchange of genetic material between two chromosomes of different colors; a dicentric chromosome (dic) contains two centromeres from chromosomes of different colors; an acentric fragment (ace) is a linear part of a red or green painted chromosome without a centromere; and an insertion (ins) is acentric chromosomal material inside the chromosome of another color. All t (one-way plus two-way), rcp (two-way) and ins were classified as stable aberrations and ace and dic as nonstable aberrations. Other analyzed parameters were the percentage of aberrant cells (%AB.C.), aberrations per 1000 cells (AB/1000) and the number of color junctions (NCJ). All cells with a color junction or a painted acentric fragment were counted as aberrant cells.

The genomic frequencies (F_G) of stable chromosome exchanges were calculated according to Lucas et al. [33] using the equation: $F_G = F_{rg}/2.05[f_r(1-f_r)+f_g(1-f_g)-f_rf_g]$. F_{rg} is the translocation frequency measured by FISH after two-color painting, while f_r (red) and f_g (green) are the fractions of the genome [34] (chromosomes #1 and #4 represent 8.28% and 6.39% of the human genome, respectively) painted red and green, respectively.

2.5. Cotinine assay and plasma lipids

Urinary cotinine levels as a marker of exposure to tobacco smoke were analyzed by radioimmunoassay [35]. The plasma levels of cholesterol, LDL- and HDL-cholesterol and triglycerides were determined spectrophotometrically using Sigma diagnostics kits and appropriate standards.

2.6. Statistical analysis

Statistical analysis was performed using SPSS software (version 17.0). Comparisons of the basic characteristics of the studied groups, the exposure to air pollutants and the levels of biomarkers in both seasons were done using the t-test for variables following a normal distribution and the Mann–Whitney test for variables that were not normally distributed. Associations between selected variables (age, cotinine, cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides, air pollutants) and the analyzed biomarkers were studied using generalized estimating equations (GEE). The type of dependent variables determined whether linear or binary logistic model was used. Oxidative stress biomarkers as continuous variables were analyzed by linear models, while cytogenetic parameters were treated as categorical variables and thus binary logistic models were used for their analysis. Multivariate analyses were adjusted to age, cotinine, cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides, and/or carbonyl levels. For binary logistic model estimates, continuous variables were transformed into a three level scale using tertiles; cytogenetic biomarkers were transformed into a two level scale using medians. To normalize the distribution of 15-F_{2t}-IsoP levels for linear models, log-transformed values were used. To correct for multiple comparisons, the FDR method was used (QVALUE software [36]).

3. Results

The average age (±standard deviation, SD) of subjects enrolled in our study was 33.0 ± 5.6 years. The average cholesterol concentration was 5.1 ± 0.8 mmol/l, levels of LDL-cholesterol reached 3.2 ± 0.6 mmol/l, HDL-cholesterol 1.4 ± 0.2 mmol/l and triglycerides 1.3 ± 0.9 mmol/l. These parameters were followed because they may affect levels of oxidative stress markers. High-fat diet results in increased levels of plasma lipids; at the same time high fat diet was shown to be associated with increased oxidative stress, including lipid peroxidation in experimental animals [37,38]. Oxidation of lipids results in formation of reactive intermediates that further propagate oxidative stress. We did not observe any significant differences between the seasons for any parameter. Exposure to tobacco smoke (measured as levels of cotinine) and environmental pollutants in February and May 2007 is shown in Table 1; for environmental pollutants the results from both personal and stationary monitors are presented. The differences between the seasons were statistically significant for exposure to both c-PAHs (including B[a]P) and PM2.5. While the concentrations of c-PAHs and B[a]P measured by both personal and stationary monitors were higher in winter than in spring, the levels of PM2.5 were unexpectedly elevated in May 2007 (mean values ± SD: 26.1 ± 14.3 and 28.4 ± 2.81 μg/m^3 for February and May 2007, respectively ($p < 0.001$)). We did not observe any significant difference between the seasons for any marker of oxidative stress or any cytogenetic parameter (Table 2). However, the levels of 15-F_{2t}-IsoP were slightly elevated in February 2007 (mean values ± SD: 0.97 ± 0.54 and 0.87 ± 0.64 nmol/mmol creatinine for February and May 2007, respectively ($p = 0.07$)). We also did not find any differences between smokers and non-smokers for any biomarker in either season (data not shown).

The following analyses were conducted among all subjects regardless of the sampling season. In Table 3 we present the results of GEE analysis of markers of oxidative stress and selected cytogenetic parameters (F_G/100, AB.C., ace) with age, cotinine, cholesterol, LDL-cholesterol, HDL-cholesterol and triglycerides. Cytogenetic parameters selected for further analyses represent those that are most commonly used and that allow standardization of results among different laboratories. Linear models revealed a negative association between 8-oxodG levels and cholesterol ($B = -0.40$, $p = 0.02$), 15-F_{2t}-IsoP and cholesterol ($B = -0.05$, $p = 0.03$), as well as between 15-F_{2t}-IsoP and LDL-cholesterol ($B = -0.07$, $p = 0.04$). Binary logistic models showed a significant effect of age on the frequency of F_G/100 ($B = 0.11$, $p < 0.01$) and %AB.C. ($B = 0.08$, $p = 0.01$),

Table 2
Comparison of the levels of oxidative stress and cytogenetic biomarkers in the winter and spring seasons.

Variable[a]	February 2007 ($N = 59$)		May 2007 ($N = 59$)		p
	Mean ± SD	Median (min, max)	Mean ± SD	Median (min, max)	
8-oxodG (nmol/mmol creatinine)	4.57 ± 1.59	4.30 (1.95, 8.63)	4.54 ± 1.91	4.57 (0.97, 9.60)	0.92
15-F_{2t}-isoprostane (nmol/mmol creatinine)	0.97 ± 0.54	0.81 (0.30, 2.75)	0.87 ± 0.64	0.70 (0.33, 3.81)	0.07
Protein carbonyls (nmol/ml)	14.1 ± 2.77	13.5 (8.81, 22.2)	14.2 ± 2.33	13.8 (10.1, 20.6)	0.73
F_G/100	1.25 ± 1.03	1.12 (0, 4.48)	1.25 ± 1.25	0.75 (0, 5.60)	0.69
%AB.C.	0.26 ± 0.16	0.20 (0, 0.60)	0.29 ± 0.23	0.20 (0, 0.90)	0.84
NCJ	3.46 ± 2.88	3.00 (0, 12.0)	3.63 ± 3.47	2.00 (0, 15.0)	0.91
AB/1000	4.14 ± 2.97	3.00 (0, 14.0)	4.61 ± 3.78	4.00 (0, 15.0)	0.81
t	3.25 ± 2.68	3.00 (0, 12.0)	3.27 ± 3.28	2.00 (0, 15.0)	0.67
rcp	1.10 ± 1.11	1.00 (0, 6.00)	1.15 ± 1.31	1.00 (0, 6.00)	0.88
dic	0.03 ± 0.18	0 (0, 1.00)	0.12 ± 0.33	0 (0, 1.00)	0.08
ace	0.44 ± 0.95	0 (0, 6.00)	0.58 ± 0.83	0 (0, 4.00)	0.15
ins	0.07 ± 0.31	0 (0, 2.00)	0.10 ± 0.30	0 (0, 1.00)	0.32

[a] 8-oxodG, 8-oxo-7,8-dihydro-2′-deoxyguanosine; F_G/100, genomic frequency of translocations/100 cells; %AB.C., percentage of aberrant cells; NCJ, color junctions/1000 cells; AB/1000, aberrations/1000 cells; t, all (one-way plus two-way) translocations/1000 cells; rcp, reciprocal (two-way) translocations/1000 cells; dic, dicentric chromosomes/1000 cells; ace, acentric fragments/1000 cells ins, insertions/1000 cells.

Table 3
Selected variables associated with oxidative stress and cytogenetic biomarkers.

	8-oxodG (B, 95% CI, p)	log 15-F_{2t}-isoprostane (B, 95% CI, p)	Protein carbonyls (B, 95% CI, p)
Age	0.02, (−0.03, 0.07), 0.38	−0.002, (−0.01, 0.01), 0.57	−0.03, (−0.12, 0.06), 0.48
Cotinine	−0.13, (−0.44, 0.18), 0.41	0.01, (−0.03, 0.05), 0.59	0.18, (−0.53, 0.68), 0.83
Cholesterol	−0.40, (−0.75, −0.06), 0.02	−0.05, (−0.10, 0.0), 0.03	0.37, (−0.12, 0.85), 0.14
LDL-cholesterol	−0.33, (−0.74, 0.07), 0.11	−0.07, (−0.13, 0.0), 0.04	0.24, (−0.55, 1.03), 0.55
HDL-cholesterol	−0.32, (−1.65, 1.01), 0.64	−0.10, (−0.29, 0.08), 0.28	1.85, (−0.25, 3.95), 0.08
Triglycerides	−0.57, (−1.88, 0.75), 0.40	−0.07, (−0.23, 0.10), 0.44	0.49, (−1.45, 2.42), 0.62
	F_G/100 (B, 95% CI, p)	%AB.C. (B, 95% CI, p)	ace (B, 95% CI, p)
Age	0.11, (0.03, 0.18); <0.01	0.08, (0.02, 0.15); 0.01	0.03 (−0.03, 0.08); 0.40
Cotinine	0.0, (0.0, 0.0); 0.56	0.0, (0.0, 0.0); 0.40	0.0, (0.0, 0.0); 0.98
Cholesterol	0.58, (0.03, 1.13); 0.04	0.45, (−0.02, 0.91); 0.06	0.27, (−0.23, 0.76); 0.29
LDL-cholesterol	0.66, (−0.12, 1.43); 0.10	0.0, (−0.55, 0.54); 0.98	0.19, (−0.44, 0.82); 0.56
HDL-cholesterol	0.17, (−1.44, 1.78); 0.84	1.44, (−0.28, 3.17); 0.10	1.90, (0.32, 3.49); 0.02
triglycerides	0.39, (−0.01, 0.80); 0.06	0.19, (−0.19, 0.56); 0.33	−0.27, (−0.74, 0.19); 0.25

an effect of cholesterol levels on the frequency of F_G/100 ($B = 0.58$, $p = 0.04$) and an effect of HDL-cholesterol on the frequency of ace ($B = 1.90$, $p = 0.02$).

We further investigated the possible relationship between oxidative stress markers and cytogenetic parameters. As evident from Table 4, we found a negative association between the levels of oxidized proteins and F_G/100, as well as ace. The effect seems to be stronger for F_G/100 (the results for the second and the third tertiles of protein carbonyl levels: $B = -1.12$, $p = 0.08$; and $B = -1.08$, $p = 0.04$, respectively). For ace, a significant association was observed only for the third tertile of protein carbonyls ($B = -1.10$, $p = 0.04$), while for %AB.C. the results for the third tertile were on the borderline of significance ($B = -1.18$, $p = 0.06$).

The results of multivariate-adjusted GEE analysis of environmental pollutants measured by both personal and stationary monitors over a 48 h period immediately preceding sample collection and markers of oxidative stress and selected cytogenetic parameters are reported in Table 5. Levels of 8-oxodG in urine were significantly elevated after exposure to PM2.5 ($B = 0.04$, $p = 0.02$), while c-PAHs and B[a]P measured by stationary monitors were associated with increased levels of 15-F_{2t}-IsoP ($B = 0.01$, $p < 0.01$ and $B = 0.03$, $p = 0.01$ for c-PAHs and B[a]P, respectively). Similar results were obtained for the association of 15-F_{2t}-IsoP with c-PAHs and B[a]P measured by personal monitors: $B = 0.01$, $p = 0.01$ and $B = 0.05$, $p < 0.01$ for c-PAHs and B[a]P, respectively. Our results indicate that none of the pollutants measured immediately before the collection of biological material directly affected the levels of cytogenetic parameters. To analyze the effect of c-PAHs and PM2.5 on changes in the levels of the studied biomarkers in more detail, we used the mean concentrations of PM2.5 and B[a]P measured by stationary monitors over various intervals before sampling as described in Section 2 and compared them with the levels of biomarkers. The significant results for both pollutants are presented in Table 6; Fig. 1 graphically demonstrates periods in which exposure to B[a]P affects the levels of biomarkers. Exposure to PM2.5 over a 14-day interval, days 15–28 before sampling, increased the frequency of acentric fragments ($B = 0.64$, $p = 0.03$). Concentrations of B[a]P measured over a 28-day interval, days 57–84 before the collection of samples, affected the levels of 8-oxodG ($B = 0.44$, $p = 0.02$) as well as the frequency of acentric fragments ($B = 0.52$, $p = 0.03$). Exposure to B[a]P over a 3-day interval immediately before the collection of samples seems to be an important factor affecting lipid peroxi-

Table 4
Association of protein oxidation and cytogenetic parameters.

Protein carbonyls (nmol/ml)	F_G/100 (B^*, 95% CI, p)	%AB.C. (B^*, 95% CI, p)	ace (B^*, 95% CI, p)
<12.92	Reference	Reference	Reference
12.92–14.65	−1.12, (−2.35, 0.12), 0.08	−0.23, (−1.54, 1.08), 0.73	−0.29 (−1.41, 0.84), 0.62
>14.65	−1.08, (−2.09, −0.08), 0.04	−1.18, (−2.40, 0.04), 0.06	−1.10 (−2.13, −0.08), 0.04

[*] Adjusted to age, cotinine, cholesterol, HDL-, LDL-cholesterol, triglycerides.

Table 5
Environmental pollutants associated with oxidative stress and cytogenetic parameters over a 2-day period before sampling.

	8-oxodG (B^*, 95% CI, p)	log 15-F_{2t}-isoprostane (B^*, 95% CI, p)	Protein carbonyls (B^*, 95% CI, p)
B[a]P (ng/m^3)[a]	0.16, (−0.22, 0.55), 0.41	0.05, (0.01, 0.09), <0.01	−0.20, (−0.78, 0.38), 0.49
c-PAHs (ng/m^3)[a]	0.02, (−0.04, 0.09), 0.46	0.01, (0.0, 0.02), 0.01	−0.05, (−0.15, 0.06), 0.39
PM2.5 (μg/m^3)[b]	0.04, (0.01, 0.07), 0.02	0.002, (0.0, 0.01), 0.27	0.0, (−0.04, 0.05), 0.99
B[a]P (ng/m^3)[b]	0.20, (−0.03, 0.42), 0.08	0.03, (0.01, 0.06), 0.01	−0.07, (−0.38, 0.23), 0.63
c-PAHs (ng/m^3)[b]	0.03, (0.0, 0.06), 0.08	0.01, (0.0, 0.01), <0.01	−0.01, (−0.05, 0.03), 0.64
	F_G/100 (B^{**}, 95% CI, p)	%AB.C. (B^{**}, 95% CI, p)	ace (B^{**}, 95% CI, p)
B[a]P (ng/m^3)[a]	0.03, (−0.44, 0.49), 0.91	0.15, (−0.33, 0.63), 0.54	−0.27, (−0.78, 0.24), 0.30
c-PAHs (ng/m^3)[a]	−0.28, (−0.80, 0.25), 0.31	0.14, (−0.41, 0.69), 0.62	−0.11, (−0.66, 0.45), 0.71
PM2.5 (μg/m^3)[b]	−0.35, (−0.91, 0.20), 0.21	−0.06, (−0.56, 0.43), 0.80	0.11, (−0.38, 0.60), 0.66
B[a]P (ng/m^3)[b]	−0.07, (−0.53, 0.39), 0.77	−0.07, (−0.51, 0.37), 0.75	−0.37, (−0.88, 0.15), 0.17
c-PAHs (ng/m^3)[b]	−0.07, (−0.53, 0.39), 0.77	−0.07, (−0.51, 0.37), 0.75	−0.37, (−0.88, 0.15), 0.17

[a] Data from personal monitors.
[b] Data from stationary monitors.
[*] Adjusted to age, cotinine, cholesterol, HDL-, LDL-cholesterol, triglycerides.
[**] Adjusted to age, cotinine, cholesterol, HDL-, LDL-cholesterol, triglycerides, protein carbonyls.

Table 6
The effect of environmental pollutants measured by stationary monitors on oxidative stress and chromosomal aberrations in other periods before sampling.

	8-oxodG			log 15-F_{2t}-isoprostane		
	Period before sampling (days)	(B^*, 95% CI, p)	q^{***}	Period before sampling (days)	(B^*, 95% CI, p)	q^{***}
B[a]P (ng/m^3)	57–84	0.44, (0.06, 0.83), 0.02	0.04	1–3	0.06, (0.01, 0.10), <0.01	<0.01
	%AB.C.			ace		
	Period before sampling (days)	(B^{**}, 95% CI, p)	q^{***}	Period before sampling (days)	(B^{**}, 95% CI, p)	q^{***}
PM2.5 (μg/m^3)				15–28	0.64, (0.05, 1.24), 0.03	0.07
B[a]P (ng/m^3)	57–70	0.73, (0.18, 1.28), <0.01	0.01	57–84	0.52, (0.06, 0.99), 0.03	0.07

[*] Adjusted to age, cotinine, cholesterol, HDL-, LDL-cholesterol, triglycerides.
[**] Adjusted to age, cotinine, cholesterol, HDL-, LDL-cholesterol, triglycerides, protein carbonyls.
[***] Correction for multiple comparisons.

dation ($B = 0.06$, $p < 0.01$). Finally, the levels of B[a]P over a 14-day interval, days 57–70 before sampling, increased the frequency of %AB.C. ($B = 0.73$, $p < 0.01$). After the correction for multiple comparisons all the results but associations of environmental pollutants with acentric fragments remained significant (q-values, Table 6).

4. Discussion

In our study we investigated the effect of environmental pollution on biomarkers of oxidative stress and cytogenetic parameters in a group of 59 city policemen in two seasons with different levels of air pollutants. We also studied possible associations between oxidative stress and cytogenetic biomarkers.

Our data indicate that there is no significant difference between the levels of the studied biomarkers in the two seasons despite differences in the concentrations of ambient air pollutants at the time of sample collection. Although exposure to c-PAHs and B[a]P measured by both personal and stationary monitors was higher in the winter season than in spring, the concentrations of PM2.5 measured by stationary monitors unexpectedly followed the opposite trend. Assuming that both pollutants affect the studied biomarkers, this situation may explain why we did not observe any difference between the analyzed parameters. Another reason may be the relatively low concentrations of environmental pollutants, particularly in the winter season, when compared with our previous studies [21,22,39]. We also cannot rule out the possibility that other chemicals that were not monitored affected levels of analyzed biomarkers. These chemicals include volatile organic compounds, metals, or ozone. Ozone has been shown to induce lipid peroxidation [40], while benzene caused oxidative DNA damage [41]. Finally, we need to take into account possible nonspecific binding of antibodies used in ELISA assays detecting oxidative stress markers to various components of urine and plasma samples.

It is believed that air pollution induces oxidative stress either directly by e.g. metals or via inflammation caused by inhaled particles, as well as by the formation of reactive quinones produced as a result of PAH metabolism. The effect of air pollution on oxidative stress markers has been analyzed in numerous studies with

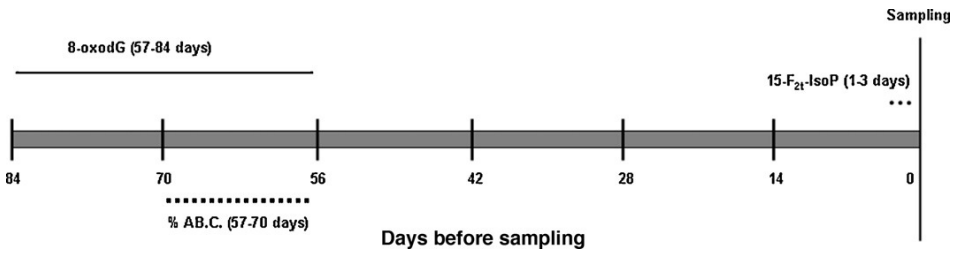

Fig. 1. The effect of B[a]P measured by stationary monitors over various periods before sampling on biomarkers of oxidative stress and cytogenetic parameters. *Abbreviations*: 8-oxodG, 8-oxo-7,8-dihydro-2′-deoxyguanosine; 15-F_{2t}-IsoP, 15-F_{2t}-isoprostane; %AB.C., percentage of aberrant cells; ace, number of acentric fragments/1000 cells

conflicting results. Urinary levels of 8-oxodG were associated with exposure to PM2.5 in a group of diesel engine exhaust emission inspectors [42] and were increased in long-distance bus drivers [43]. Exposure to PAHs and PM2.5 increased urinary levels of 8-oxodG in security guards working at a gate by a busy road after their working shift [44]. In highway toll station workers, levels of 8-oxodG in urine were higher than those in a control group [45]. On the other hand, oxidative stress markers (8-oxodG, 15-F_{2t}-IsoP, protein carbonyls) were not associated with exposure to PM2.5 in a group of New York City subway workers, probably reflecting the different compositions of PM arising mainly from subway train traffic [46]. In our previous studies [39,47], oxidative damage to DNA, lipids and proteins was elevated in a group of bus drivers when compared with controls even though exposure to environmental pollutants (c-PAHs, B[a]P) measured by personal monitors was higher in the control group in some seasons. In these studies, we observed seasonal variability of oxidative stress markers, although these differences were not significant for all comparisons. Our present results do not confirm those data even though the concentrations of pollutants differed significantly between the sampling seasons. As mentioned above, we believe that one of the explanations for this result is the opposite trends found for the concentration changes of c-PAHs and PM2.5. We may speculate that since both groups of pollutants may induce oxidative stress, potential differences in the levels of oxidative stress biomarkers were eliminated.

In our previous studies we identified exposure to PM2.5 and PM10 over a 3-day period before sample collection as the only factors affecting 8-oxodG levels [39] and exposure to B[a]P and c-PAHs in the same period as factors increasing the levels of lipid peroxidation [47]. The results of the present study are mostly in agreement with these data, although we also identified a period in which exposure to B[a]P induces 8-oxodG levels. We may conclude that PM2.5 is a more potent inducer of oxidative damage to DNA than B[a]P because urinary levels of 8-oxodG increase already 2–3 days after exposure to PM2.5. Before inducing oxidative stress, B[a]P needs to be metabolized via an alternative metabolic pathway to *o*-quinones. Therefore, a longer period after exposure may be necessary to result in the induction of 8-oxodG and its excretion in urine.

For studies of the effect of various pollutants on genetic damage, conventional cytogenetic analysis or the analysis of micronuclei (MN) is most often used. Thus, genetic damaged has been studied in subjects processing electronic waste [48], exposed to environmental tobacco smoke [49], organic solvents [50] and PM and other environmental pollutants [30,51,52]. Only in several studies was FISH used to assess the frequency of chromosomal aberrations [21,22,53–55], although based on a comparison with conventional techniques, FISH seems to be more sensitive for monitoring exposure to air pollution. In several studies [21,22,53], the frequencies of CA were significantly higher in exposed individuals than in controls. In the present study we did not observe any differences in the levels of CA between the two seasons. As mentioned before, we believe the lack of a significant difference is due to the low concentrations of environmental pollutants in the winter season. While in previous studies exposure to B[a]P in exposed groups ranged from 1.58 to 4.52 ng/m^3, in the present study the average exposure to B[a]P in the winter season was 1.03 ng/m^3. Our results suggest that this level of B[a]P is not sufficient to induce significant genetic damage; it is probably borderline for biological effects of this compound. A previous study showed that for the induction of CA, a critical period for exposure to B[a]P is a 10-day interval, days 40–50 before sampling [21]. According to our data, the frequency of aberrant cells is affected by exposure to B[a]P over a 14-day period, 57–70 days before the collection of samples. Although the critical period is not exactly the same in both studies, we can conclude that in general, CA are induced by about a 2-week exposure to B[a]P about two months before sample collection. It should be noted that the critical period probably depends not only on the time frame before sample collection, but also on the concentrations of B[a]P during this time. In our study, the mean concentration of B[a]P on days 57–70 before sampling (mean ± SD: 2.15 ± 0.72 μg/m^3) was the highest of all concentrations measured over 14-day intervals up to 84 days before the collection of samples. Moreover, this concentration is substantially higher than 1 ng B[a]P/m^3, a concentration that is probably sufficiently low not to induce genetic changes [30,53,56]. Aberrant cells probably arise from the accumulation of PAH–DNA adducts formed after the metabolic activation of PAHs, although oxidative stress induced by the formation of reactive quinones may also play a role. It is probable that the induction of inflammation is a faster process than the accumulation of PAH–DNA adducts and the formation of CA as a result of errors during DNA repair.

Our results suggest that higher levels of protein carbonyl groups in blood plasma are associated with lower frequency of chromosomal aberrations. This effect was particularly prominent for the frequency of F_G/100. Serum albumin, the most abundant protein in blood plasma, has been identified as an important antioxidant that protects the organism against the effect of molecules with pro-oxidant properties (reviewed in [57]). It binds cationic ligands, including transition metals, as well as lipids and scavenges hydroxyl radicals. Hydroxyl radicals cause protein fragmentation and the formation of carbonyl groups [58]. Thus, we speculate that increased carbonyl levels, a consequence of the radical-scavenging properties of serum albumin, decrease the probability of the formation of CA because serum albumin traps hydroxyl radicals that would otherwise cause chromosomal breaks. It is evident that only CA induced as a result of oxidative stress may be prevented by this mechanism. To the best of our knowledge this is the first report of the possible beneficial effects of protein oxidation on genetic damage caused to chromosomes. However, to rule out the possibility that this is a spurious finding, the results should be replicated in larger groups in other studies.

We should also mention associations between plasma concentrations of cholesterol, LDL- and HDL-cholesterol, and triglycerides and biomarkers of oxidative stress (8-oxodG, 15-F_{2t}-IsoP) and cytogenetic parameters (F_G/100, %AB.C., ace). While a positive association was observed between cytogenetic parameters (particularly F_G/100) and cholesterol thus confirming our previous results [21], oxidative stress markers showed opposite trends. Currently, we do not have any explanation for this unexpected observation. Again, we cannot rule out the result is a spurious finding.

In our study, we did not observe seasonal variability in any marker of oxidative stress or any cytogenetic parameter. We confirmed the time periods before sampling that are critical for the urinary excretion of 8-oxodG by PM2.5 and lipid peroxidation and chromosomal damage by B[a]P. For the first time we show that exposure to B[a]P might increase 8-oxodG levels. Also for the first time, we report the possible protective effect of protein oxidation on the formation of chromosomal aberrations. In conclusion, our study demonstrates that environmental pollutants affect urinary excretion of 8-oxodG, lipid peroxidation and chromosomal aberrations.

Conflict of interest statement

The authors have no conflict of interest to disclose.

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Příloha 4:

R.J. Sram, B. Binkova, O. Beskid, A. Milcova, P. Rossner, P. Rossner Jr., A.Rossnerova, I. Solansky, J. Topinka

Biomarkers of exposure and effect – interpretation in human risk assessment

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Biomarkers of exposure and effect—interpretation in human risk assessment

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Abstract The effect of exposure to carcinogenic polycyclic aromatic hydrocarbons adsorbed onto respirable air particles (PM2.5, diameter <2.5 μm) on DNA adducts and chromosomal aberrations was repeatedly studied in Prague, Czech Republic, in groups of policemen working in the downtown area and in bus drivers. Personal exposure was evaluated using personal samplers during working shifts. DNA adducts were analyzed in lymphocytes by the ^{32}P-postlabeling assay and chromosomal aberrations were analyzed by conventional cytogenetic analysis and fluorescent in situ hybridization (FISH). The impact of environmental pollution on DNA adducts and chromosomal aberrations was studied in a total of 950 subjects. Our results suggest that the environmental exposure of nonsmokers to concentrations higher than 1 ng benzo[a]pyrene/m^3 represents a risk of DNA damage, as indicated by an increase in DNA adducts and the genomic frequency of translocations determined by FISH.

Keywords Personal monitoring · Carcinogenic polycyclic aromatic hydrocarbons · Benzo[a]pyrene · DNA adducts · Chromosomal aberrations

Introduction

Epidemiological studies have shown that prolonged exposure to particulate air pollution may be associated with an increased rate of morbidity and mortality from respiratory and cardiovascular diseases in the general population. Polycyclic aromatic hydrocarbons (PAHs) adsorbed onto respirable air particles (PM2.5, diameter <2.5 μm) are mainly derived from incomplete combustion, including mobile sources, such as motor vehicles, and stationary sources, such as power plants, residential heating, among others. Some of these compounds exhibit carcinogenic and/or mutagenic properties (Lewtas 2007). Molecular epidemiology studies using biomarkers of exposure and early biological effects could provide invaluable information on the genotoxic effects of environmental exposure to such PAH mixtures. One promising biomarker seems to be the measurement of DNA adducts, since such measurements take into account individual differences in exposure, absorption, distribution, metabolic activation, and detoxification of PAHs in the body as well as cell turnover and the repair of DNA damage (Binkova et al. 2007a; Lewtas 2007).

Chromosomal aberrations in human peripheral lymphocytes are recognized as a valuable biomarker of effect, probably the only such biomarker that has been standardized and validated at the international level (Bonassi et al. 2008). While classic cytogenetic analysis (conventional method) is the method of choice for determining unstable types of aberrations, the fluorescent in situ hybridization technique (FISH) seems to be a rapid, sensitive, and reliable method for the detection of stable structural rearrangements that remain undiminished over time, such as translocations (Rubes et al. 1998; Verdorfer et al. 2001; Tucker et al. 2003; Sram et al. 2007a; Sigurdson et al. 2008). The FISH painting technique appears to be more sensitive than the conventional technique for detecting the genomic frequency of translocations induced by various chemical agents or irradiation (Sram et al. 2007b).

The capital city of Prague has become one of the most polluted localities in the Czech Republic, primarily due to

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traffic. Therefore, the effect of exposure to carcinogenic PAHs (c-PAHs) adsorbed onto respirable air particles (on DNA adducts and chromosomal aberrations was repeatedly studied in groups of policemen working in the downtown area and in bus drivers.

Methods

The study cohort comprised 950 individuals from three studies; the subjects of the analyses were categorized into the following groups: (1) policemen in 2001 (exposed n=53, controls n=52) (Sram et al. 2007a); (2) policemen in 2004 (exposed n=480, 120×4) (Topinka et al. 2007); (3) bus drivers in 2005–2006 (n=120 × 3; exposed I n=50, exposed II n=20, controls n=50) (Rossner et al. 2008). All volunteers were male city policemen working in downtown Prague and spending >8 h outdoors daily. Controls from study (1) were age- and sex-matched healthy males spending >90% of their daily time indoors and working in a suburban area. Study (3) involved 50 bus drivers working in the center of Prague (I), 20 garage men (II) and, as controls, 50 healthy administrative workers spending >90% of their daily time indoors (Table 1).

Ambient air particles (PM10, PM2.5) and c-PAHs, namely, benz[a]anthracene, benzo[b]fluoranthene, benzo [k]fluoranthene, benzo[ghi]perylene, benzo[a]pyrene (B[a] P), chrysene, dibenz[ah]-anthracene, and indeno[cd]pyrene, were monitored using VAPS samplers, while personal exposure was evaluated using personal samplers during working shifts. Quantitative chemical analysis of c-PAHs was performed by high-performance liquid chromatography (HPLC) with fluorimetric detection according to the U.S. Environmental Protection Agency method.

DNA adducts were analyzed in lymphocytes by the ^{32}P-postlabeling assay, which was performed according to a standardized procedure (Fig. 1) (Binkova et al. 2007a). Briefly, DNA samples (6 μg) were digested by a mixture of micrococcal endonuclease and spleen phosphodiesterase for 4 h at 37°C; P1 nuclease was used for adduct enrichment. The labeled DNA adducts were resolved by two-directional thin layer chromatography on 10 × 10-cm PEI-cellulose plates. Three solvent systems were used for thin layer chromatography (TLC): D-1 (1 M sodium phosphate, pH 6.8); D-2 (3.8 M lithium formate, 8.5 M urea, pH 3.5); D-3 (0.8 M lithium chloride, 0.5 M Tris, 8.5 M urea, pH 8.0). Autoradiography was carried out at −80°C for 72–120 h. A diagonal radioactive zone and/or distinct DNA adduct spots were excised using the same template for all samples. The ^{32}P-radioactivity was measured by liquid scintillation counting. To determine the exact amount of DNA in each sample, aliquots of the DNA enzymatic digest (0.5 μg of DNA hydrolysate) were analyzed for nucleotide content by reverse-phase HPLC with UV detection, which simultaneously allowed for controlling the purity of the DNA. DNA adduct levels were expressed as adducts per 10^8 nucleotides. A B[a]P diolepoxide-DNA adduct standard was run in triplicate in each postlabeling experiment to

Table 1 Overview of the study data

Biomarkers	Policemen 2001				Policemen 2004	Bus driver 2005		
	Exp SM	Exp NS	Con SM	Con NS	NS	Exp[a] NS	Exp[b] NS	Con NS
n	29	24	11	41	89	49	19	45
Age (years)	33.1±6. 9	29.9±7.3	35.6±12.3	27.9±7.5	34.4±8.3	49.5±9.6	35.8±10.2	50.1±10.9
B[a]P (ng/m^3)	2.2±2.0	1.4±1.0	0.6±0.5	0.9±0.6	1.5±1.3	1.3±0.7	3.4±4.1	1.7±1.0
c-PAH (ng/m^3)	14.3±13.2	9.4±7. 6	4.7±2.2	6. 6±3. 7	8.6±9.0	7.2±3.7	19.4±24.1	9.1±5.6
Vitamin A (μmol/l)	2.2±0.6	1.8±0.6	2.2±0.8	1.8±0.5	3.5±1.0	3.4±0.9	2.8±0.6	3.0±0.8
Vitamin E (μmol/l)	26.7±9.2	25.0±10.9	33.5±29.0	24.5±6.6	24.1±8.8	32.1±8.6	23.7±6.3	23.0±7.1
Vitamin C (μmol/l)	84.2±40.3	86.4±53.1	60.0±28.1	98.3±58.6	64.4±15.9	52.2±19.6	64.3±14.7	56.8±18.7
Folate (μmol/l)	18.04±11.59	17.51±9.90	13.48±7.52	17.48±9.04	25.48±18.31	-	-	-
Cotinine/creatinine (ng/mg)	1714±1563	21±11	2072±1901	16±11	16±9	16±11	13±8	12±9
Cholesterol (mmol/l)	4.2±1.0	3.3±0.9	4.1±1.0	3.6±0.7	3.9±1.2	5.9±1.1	5.8±1.2	5.5±0. 9
HDL cholesterol (mmol/l)	0.8±0.2	0.9±0.2	0.9±0.3	1.0±0.3	1.0±0.3	1.4±0.3	1.5±0.2	1.5±0.2
LDL cholesterol (mmol/l)	2.7±0.8	2.2±0.7	2.6±0.9	2.2±0.6	2.2±0.8	3.5±0.8	3.3±0.8	3.2±0.7
Triglycerides (mmol/l)	2.2±1.9	1.3±0.9	2.1±1.5	1.1±0.7	1.4±1.0	2.0±1.1	2.0±1.4	2.1±1.7

Exp SM, Exposed smokers; Exp NS, exposed nonsmokers; Con SM, control smokers; Con NS, control nonsmokers; B[a]P, benzo[a]pyrene; c-PAHs, carcinogenic polycyclic aromatic hydrocarbons; HDL, high-density lipoprotein; LDL, low-density lipoprotein

[a] Bus drivers

[b] Garage men

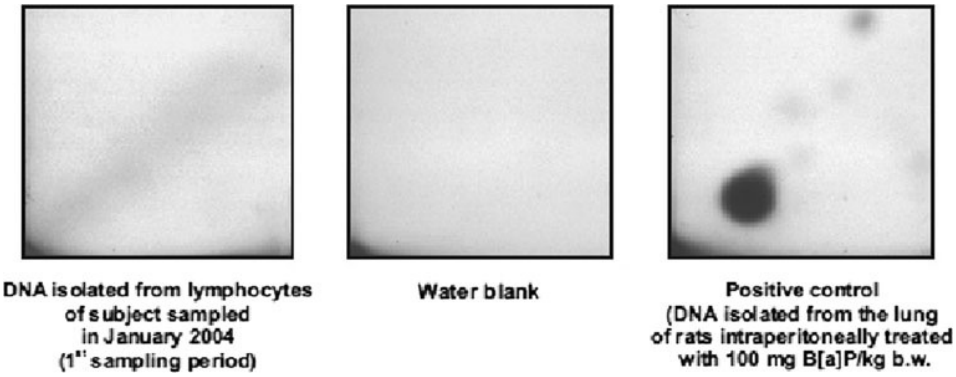

Fig. 1 DNA adducts by ^{32}P-postlabeling. *B[a]P* Benzo[a] pyrene, *b.w.* body weight

control for interassay variability and to normalize the calculated DNA adduct levels. The data presented here are average values from three independent experiments (variability of the total and "like-B[a]P"-DNA adduct levels for each: less than ±20%).

Chromosomal aberrations were analyzed by conventional cytogenetic analysis and by FISH whole chromosome painting for chromosomes no. 1 and no. 4 (Fig. 2) (Sram et al. 2007b).

For the conventional cytogenetic analysis (CCA), a suspension from the last fixation step was dropped onto slides, and the slides were air-dried and stained with 5% Giemsa solution (pH 6.8). The slides from each culture of peripheral blood lymphocytes were numbered randomly and scored "blind" in numerical order. One hundred well-spread metaphases with 46±1 centromeres were examined per donor. The following were evaluated as chromosomal aberrations: chromatid and chromosome breaks, and chromatid and chromosome exchanges.

The FISH analysis was performed using commercial WCP (whole chromosome painting) probes differing in color (Cambio, Cambridge, UK) for chromosomes no. 1 (biotinylated) and no. 4 [fluorescein isothiocyanate (FITC)-labeled] according to the manufacturer´s chromosome painting protocol (Rubes et al. 1998). One thousand metaphases were examined for each subject under a fluorescent microscope equipped with a triple-band pass filter for visualization of 4',6-diamidino-2-phenylindole (blue), FITC (green), and cyanine (red) signals.

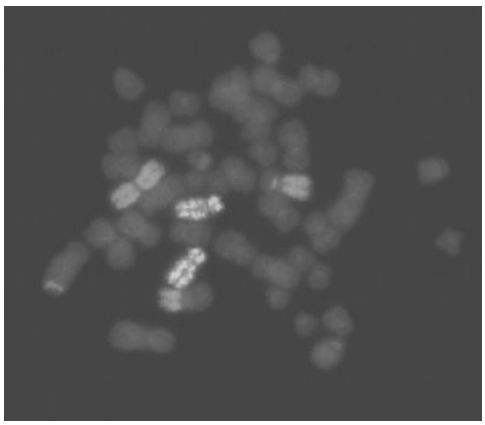

Fig. 2 Chromosomal aberrations by fluorescence in situ hybridization (FISH): whole chromosome painting #1 and #4. Shown are (t(Ab), t (Ab), and t(Ba), three translocations between chromosome 1 and unpainted chromosomes

Aberrant cells were classified according to the Protocol for Aberration Identification and Nomenclature (PAINT) (Tucker et al. 1995) and recorded by ISIS 4.4.16 software (MetaSystem GmbH, Frankfurt, Germany) as translocations, reciprocal translocations, dicentric chromosomes, acentric fragments, and insertions. Other analyzed parameters were the percentage of aberrant cells (% AB.C.), aberrations per 1000 cells (AB/1000), and the number of color junctions. The genomic frequencies (F_G) of stable chromosomal exchanges were calculated according to Lucas and Sachs (Lucas and Sachs 1993) using the equation: $F_G=F_{rg}/2.05\ [f_r(1-f_r)+f_g(1-f_g)-f_rf_g]$. F_{rg} is the translocation frequency measured by FISH after two-color painting, and f_r and f_g are the fractions of the genome painted red and green, respectively. Additional biomarkers included cotinine in urine determined by radioimmunoassay, plasma levels of vitamins A, E, and C by HPLC, folic acid by enzyme-linked immunosorbent assay (ELISA), and cholesterol and triglycerides using commercial kits; polymorphisms of metabolic genotypes [GSTM1, GSTP1, GSTT1, EPHX1, cytochrome 450 1A1-MspI (CYP1A1-MspI)] and DNA repair genotypes (XRCC1 and XPD) were determined by PCR-based restriction fragment length polymorphism (RFLP) assays.

Statistical analysis

Statistica (StatSoft, Tulsa, IK) multiple regression and SAS (SAS Institute, Cary, NC) logistic regression were used to model the association between DNA-adduct levels and variables in terms of exposure, potential modifiers of effects, and confounders . Generally, a level of $p=0.10$

was used for including a variable into the multivariate model (PIN) and a level of $p=0.15$ was used for removing a variable (POUT).

Bivariate and multivariate logistic regression were performed to identify the impact of the monitored markers on the level of genetic damage. For logistic regression estimates, variables were transformed into a three level scale using tertiles or into a two level scale using medians.

Results

Based on the personal monitoring data, during their working shifts the policemen were exposed to significantly higher concentrations of both c-PAHs and B[a]P than were the controls (median): 9.7 vs. 5.8 ng/m^3 ($p<0.01$) and 1.6 vs 0.8 ng/m^3 ($p<0.01$), respectively The level of "like" B[a]P-derived DNA adducts was higher in the exposed group than in the controls (0.122±0.036 vs. 0.099±0.035 adducts/10^8 nucleotides, $p=0.003$). The results of multivariate regression analysis showed smoking, vitamin C levels, and polymorphisms of the XPD repair gene in exon 23 and the GSTM1 gene to be significant predictors for total DNA adduct levels. Exposure to ambient air pollution, smoking, and polymorphisms of the XPD repair gene in exon 6 were significant predictors for B[a]P-"like" DNA adducts (Binkova et al. 2007a). Using the FISH technique and probes for chromosomes no. 1 and no. 4, the genomic frequency of translocations calculated as $F_G/100$ was 1.72 and 1.24 for EXP and CON ($p<0.05$), respectively. The CYP1A1*2 C (Ile/Ile), XPD 23 (Lys/Lys), and XPD 6 (CC) genotypes were associated with an increase in the number of aberrant cells, as determined by the conventional method. Factors associated with an increased level of translocations determined by FISH included age, smoking, B[a]P-like DNA adducts (corresponding to exposure to c-PAHs), folate, and polymorphisms of CYP1A1*2 C, GSTP1, EPHX1, p53 MspI, and MTHFR. Ambient air exposure to c-PAHs significantly increased FISH cytogenetic parameters in nonsmoking policemen (Sram et al. 2007a).

Total DNA adducts, B[a]P-"like" DNA adducts, and the genomic frequency of translocations were significantly affected by smoking—an effect of air pollution was observed only in nonsmokers (Binkova et al. 2007a; Sram et al. 2007a). Therefore, later studies used only nonsmokers as volunteers.

The obtained results were confirmed in a subsequent study in which city policemen were sampled in January, March, June, and September. Using a personal monitoring approach, the concentrations of c-PAHs were 1.58 and 9.07 ng/m^3 for B[a]P and c-PAHs during January, 0.39 and 3.46 ng/m^3 for B[a]P and c-PAHs during March, 0.18 and 1.92 ng/m^3 for B[a]P and c-PAHs during June, and 0.45 and 3.08 ng/m^3 for B[a]P and c-PAHs during September. Total DNA adducts were only slightly elevated in January (2.08±1.60) compared to March (1.66±0.65), June (1.96±1.73), and September (1.77±1.77). B[a]P-like DNA adducts, however, were significantly higher in January than in the March and June sampling periods (0.26±0.14 vs. 0.19±0.12 and 0.22±0.13, respectively; $p<0.0001$ and $p=0.017$), indicating that c-PAH exposure probably plays a crucial role in DNA adduct formation in lymphocytes (Topinka et al. 2007). In those same periods, the mean frequency of translocations measured by FISH ($F_G/100$) was 1.32±1.07, 0.85±0.95, 0.87±0.81, and 1.08±0.94, respectively, and the frequency of chromosomal aberrations determined by CCA was 2.07±1.48, 1.84±1.28, 1.84±1.42, and 1.64±1.46% AB.C., respectively.

In another study, bus drivers were sampled in the winter of 2005, summer of 2006, and winter of 2006. Using the personal monitoring approach, the concentrations of B[a]P for the exposed group were 1.25, 0.20, and 1.04 ng/m^3 during the winter of 2005, summer of 2006, and winter of 2006, respectively; for the controls, the concentrations were 1.75, 0.24, and 0.75 ng/m^3, respectively, during these same periods. The total DNA adducts in the exposed group were 1.72±0.56,1.22±0.45, and 1.62±0.59 adducts/10^8 nucleotides during the winter of 2005, summer of 2006, and winter of 2006, respectively; for garage men, the total DNA adducts were 1.24±0.41, 1.27±0.48, and 1.70±0.08 adducts/10^8 nucleotides during the winter of 2005, summer of 2006, and winter of 2006, respectively; in controls, the totals were 2.15±0.61, 1.18±0.36, and 1.90±0.79 adducts/10^8 nucleotides during the winter of 2005, summer of 2006, and winter of 2006, respectively. In these same three periods, the mean frequencies of translocations measured by FISH ($F_G/100$) were 1.62±1.17, 2.18±1.75, and 1.77±1.31 in the group of bus drivers, 1.20±1.24, 0.88±1.11, and 1.01±0.78 in garage men, and 1.65±149, 1.34±1.01, and 1.87±1.29 in controls, respectively. The frequencies of chromosomal aberrations determined by CCA in these three periods were 1.30±1.15, 1.43±1.01, and 1.30±1.04 % AB.C. in the group of bus drivers,0.95±0.76, 1.15±1.09, and 1.55±0.97 % AB.C. in garage men, and 1.17±0.93, 1.50±0.99, and 1.52±1.12 % AB.C. in controls, respectively.

The levels of DNA adducts were significantly affected by stationary exposure to B[a]P within the last 30 days (Table 2). Data obtained for biomarkers of exposure and effect were used for a pooled analysis. Using multivariate logistic regression, we calculated the relationship between personal exposure to B[a]P and DNA adducts (DNA adducts=1.042+B[a]P x 0.077,$p<0.001$; Fig. 3). These results indicate that c-PAH exposure plays a crucial role in DNA adduct formation in lymphocytes. A similar relationship was observed between personal exposure to B[a]P and

Table 2 Multivariate impact of environmental pollution to DNA adducts (nonsmokers, period 15 days)

Variable	Period (days)	Impact to DNA adducts	
		c-PAHs	B[a]P
Intercept	1-15	1.150	1.135
Environmental pollution[a] (ng/m^3)		0.012 (p=0.0578)	0.068 (p=0.0189)
Vitamin A (μmol/l)		0.133 (p=0.0000)	0.135 (p=0.0000)
GSTM1 (null/positive)		-0.157 (p=0.0355)	-0.159 (p=0.0332)
Intercept	16-30	1.212	1.166
Environmental pollution[a] (ng/m^3)		0.007 (p=0.31)	0.058 (p=0.0780)
Vitamin A (μmol/l)		0.127 (p=0.0000)	0.132 (p=0.0000)
GSTM1 (null/positive)		-0.154 (p=0.0388)	-0.157 (p=0.0354)

[a] Environmental pollution: c-PAHs or B[a]P

the genomic frequency of translocations measured by FISH (F_G/100=1.255+B[a]P × 0.082, p<0.05; Fig. 4). Figures 3 and 4 show the quasi-linear dose response impact of pollution levels from personal monitoring to DNA adducts and FISH F_G/100 estimated by multivariate linear regression, respectively.

Discussion

Studying the ambient concentrations of c-PAHs needed to induce DNA adducts and the genomic frequency of translocations measured by FISH, we observed that the impact of active smoking was more significant than the effect of air pollution. Therefore, we propose to use only nonsmokers in future studies.

The average personal environmental exposure to approximately 10 ng/m^3 of c-PAHs (or 1.6 ng/m^3 of B[a]P, or approx. twofold higher than the c-PAHs concentrations measured by stationary monitoring—based on the according to outdoor/indoor ratio) during the winter increased DNA adduct formation and decreased repair efficiency, which may be further affected by genetic polymorphisms. This concentration of c-PAHs and its impact on increased DNA adduct formation seems to be important for re-evaluating the risk assessment of c-PAHs. PAH-DNA adduct formation represents one of the key first steps in carcinogenesis (Gammon et al. 2004). According to Phillips (2005), the use of DNA adducts as a measure of exposure, several years prior to the onset (or clinical manifestation) of disease, can help identify individuals at a higher probability of subsequently developing cancer. Another study demonstrated significant correlations between DNA adduct levels and atherosclerosis (Binkova et al. 2002). Both examples demonstrate the potential of DNA adducts as biomarkers of risk.

DNA adducts determined by the ^{32}P-postlabeling method are sensitive biomarkers of environmental exposure to c-PAHs, if a study simultaneously includes personal and stationary monitoring, information on life style, determination of cotinine, vitamin and lipid levels, and genetic polymorphisms of metabolic and DNA repair genes.

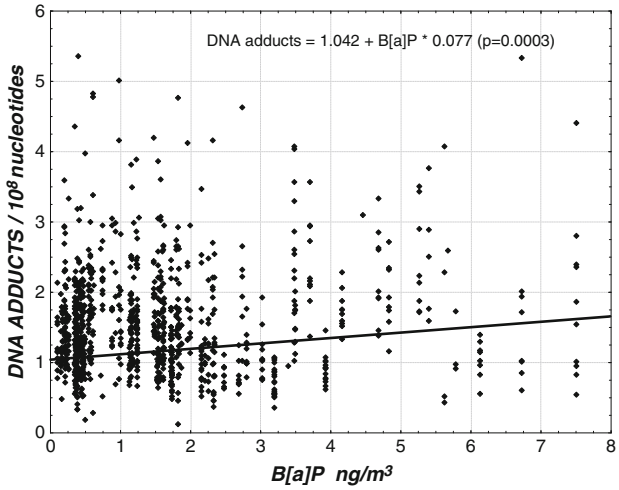

Fig. 3 Impact of B[a]P exposure to DNA adducts

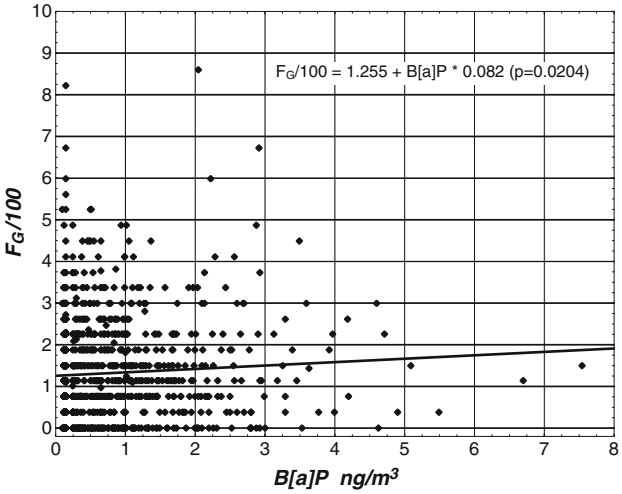

Fig. 4 Impact of B[a]P exposure to genomic frequency of translocations (F_G/100)

It is currently accepted that a high frequency of chromosomal aberrations in peripheral lymphocytes is predictive of an increased risk of cancer (Bonassi et al. 2008). Therefore, we may hypothesize that environmental exposure to c-PAHs that increases the genomic frequency of translocations represents a significant health risk. Surprising results were observed in the group of city policemen who were examined in January and March: the genomic frequency of translocations decreased in a similar manner as did their exposure to c-PAHs. This result reveals that these chromosomal aberrations are not so stable as originally expected. When we studied the relationship between chromosomal aberrations and DNA adducts in the same subjects using FISH, multiple regression analysis indicated that B[a]P-like DNA adducts are a significant predictor of the genomic frequency of translocations (Binkova et al. 2007a). Whole chromosome painting using the FISH technique is more sensitive than the originally used conventional cytogenetic method, which was not affected by the studied concentrations of c-PAHs.

Carcinogenic PAHs are adsorbed on fine respiratory particles (PM2.5) and probably represent the most important biologically active group of pollutants (Binkova et al. 2007b). As c-PAHs were not originally listed among those chemicals whose concentrations should be monitored, stationary monitoring of c-PAHs was started in some European countries only recently. It has been proposed by the EU that the emission limit for B[a]P concentration in the ambient air should be 1 ng /m^3/year. Our data indicate that such a concentration can still induce genetic injury. Certainly a concentration of a chemical carcinogen that is able to induce translocations should be considered as deleterious.

Chromosomal aberrations detected as translocations by FISH are a sensitive biomarker of effect and can help to evaluate the risk of occupational and environmental exposure to mutagens and carcinogens. Our FISH results provide new knowledge about the risk of c-PAHs in polluted air, which may significantly affect human health. This new knowledge should be used for risk assessment and to determine new standards for long-term environmental c-PAHs exposure.

Conclusions

The results of our studies suggest that environmental exposure to concentrations >1 ng B[a]P/m^3 represent a risk of DNA damage, as indicated by an increase in DNA adducts and an increase in translocations detected by FISH. Among our study cohort, the level of DNA adducts was significantly affected by B[a]P exposure within the last 30 days.

This is the first published report of a relationship between DNA adducts (biomarker of exposure) and chromosomal aberrations as determined by FISH (biomarker of effect).

Our results also suggest that DNA adducts in the lymphocytes of subjects exposed to increased c-PAH levels are an appropriate biomarker of a biologically effective dose, directly indicating whether or not the extent of exposure to these compounds is related to an increased mutagenic and carcinogenic risk. All results indicate that molecular epidemiology studies should be done in a very complex manner, simultaneously using biomarkers of exposure, effect, and susceptibility. Air pollution, and specifically c-PAHs, induce genetic damage and may significantly affect human health.

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Příloha 5:

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Expression of *XRCC5* in peripheral blood lymphocytes is upregulated in subjects from a heavy polluted region in the Czech Republic

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Expression of *XRCC5* in peripheral blood lymphocytes is upregulated in subjects from a heavily polluted region in the Czech Republic

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ABSTRACT

Air pollution causes oxidative damage to macromolecules, chromosomal aberrations and changes in gene expression. We investigated the levels of oxidative stress markers [8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG), 15-F_{2t}-isoprostane (15-F2t-IsoP), protein carbonyls] and cytogenetic parameters [genomic frequency of translocations (F_G/100), percentage of aberrant cells (%AB.C.) and acentric fragments (ace)] in subjects living in Prague and in the heavily polluted Ostrava region. We also compared the expression of genes participating in base excision repair (BER) and non-homologous end-joining (NHEJ). We analyzed 64 subjects from Prague and 75 subjects from Ostrava. We measured oxidative stress markers by ELISA, cytogenetic parameters by fluorescence in situ hybridization and gene expression by quantitative PCR. The levels of air pollutants (benzo[a]pyrene, B[a]P; carcinogenic polycyclic aromatic hydrocarbons, c-PAHs; benzene) measured by personal monitors were significantly elevated in Ostrava compared to Prague ($p < 0.001$). Despite this fact, we observed no differences in biomarkers of oxidative stress between the two locations. Moreover, subjects from Ostrava were less likely to have above-median levels of %AB.C. (OR; 95% CI: 0.18; 0.05–0.67; $p = 0.010$). Multivariate analyses revealed that subjects living in Ostrava had increased odds of having above-median levels of *XRCC5* expression (OR; 95% CI: 3.33; 1.03–10.8; $q = 0.046$). Above-median levels of 8-oxodG were associated with decreased levels of vitamins C (OR; 95% CI: 0.37; 0.16–0.83; $p = 0.016$) and E (OR; 95% CI: 0.25; 0.08–0.75; $p = 0.013$), which were elevated in subjects from Ostrava. We suggest that air pollution by c-PAHs affects *XRCC5* gene expression, which probably protects subjects from Ostrava against the induction of a higher frequency of translocations; elevated vitamin C and E levels in the Ostrava subjects decrease the levels of 8-oxodG.

1. Introduction

Ambient air pollution poses a problem to millions of people worldwide. Exposure to polluted air is associated with increased mortality and a higher incidence of cardiovascular and pulmonary diseases, as well as cancer [1]. Among many chemical compounds present in the ambient air, polycyclic aromatic hydrocarbons (PAHs) and benzene are notable because of their carcinogenicity and their abundance in the environment [2]. PAHs require metabolic activation to exert their mutagenic and/or carcinogenic effects. Three principal pathways are currently proposed for the metabolic activation of PAHs: the pathway via dihydrodiol epoxide, the pathway via radical cation by one-electron oxidation and the *o*-quinone pathway [3]. Reactive intermediates formed as a result of the metabolic activation of PAHs may bind to DNA, form PAH-DNA adducts, cause mutations and thus increase cancer risk [4]. The *o*-quinone pathway of PAH metabolism leads to redox cycling, the formation of reactive oxygen species (ROS) and thus oxidative damage of cellular macromolecules [5]. Benzene is metabolized in the liver by CYP2E1 into benzene oxide, which is further metabolized into several products, including reactive quinones [6] that again may induce oxidative damage.

Oxidative damage affects all macromolecules: DNA, lipids and proteins [7]. Damage caused to DNA results in single- or double

Abbreviations: 8-oxodG, 8-oxo-7,8-dihydro-2′-deoxyguanosine; ACTB, actin β; APE1/APEX1, apurinic/apyrimidinic endonuclease; B[a]P, benzo[a]pyrene; BER, base excision repair; c-PAHs, carcinogenic polycyclic aromatic hydrocarbons; Cq, quantification cycle; DSB, double strand break; ELISA, enzyme-linked immunosorbent assay; ETS, environmental tobacco smoke; FISH, fluorescence in situ hybridization; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NHEJ, non-homologous end-joining; OGG1, 8-oxoguanine DNA glycosylase; 15-F2t-IsoP, 15-F_{2t}-isoprostane; LIG4, ligase 4; qPCR, quantitative PCR; XRCC, X-ray repair complementing defective repair in Chinese hamster cells.

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strand breaks (DSB) of the nucleic acid chain or base modifications leading to mutations [8,9]. 8-Oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) is the most abundant and most frequently studied oxidatively modified DNA base. If unrepaired, its presence in DNA leads to GC>TA transversions. Oxidized bases in DNA, including 8-oxodG, are repaired by the action of the short-patch or long-patch base excision repair pathway (BER) [10]. The first step of BER involves a DNA glycosylase that cleaves the modified base, which results in an apurinic/apyrimidinic (AP) site formation. Mammalian cells contain eleven different glycosylases, each with a specialized function [11]; 8-oxoguanine DNA glycosylase (OGG1) was reported to be responsible for 90% of glycosylase activity in human cell extracts [12]. The AP site is recognized and cleaved by apurinic/apyrimidinic endonuclease (APE1/APEX1), the missing nucleotide is replaced by the activity of DNA polymerase β (Pol β) and DNA is sealed by the DNA ligase III/XRCC1 complex. In the event of long-patch repair, several nucleotides are replaced by the action of the proteins Pol β, PCNA, Fen 1 and ligase 1 [10].

DNA strand breaks are considered one of the most dangerous DNA lesions [13]. They can induce apoptosis, cause gene inactivation, or lead to chromosomal aberrations [14]. DNA strand breaks are repaired by two mechanisms: DNA ends that share long homologous regions are joined by homologous recombination, while non-homologous end-joining repair (NHEJ) occurs when DNA strands with as few as 2 bp of homology are ligated. NHEJ is believed to be a pathway for the repair of most double-strand DNA breaks involved in chromosomal translocations [15]. A number of proteins take part in the recognition of double-strand breaks and their repair [15]. The first protein that binds at DNA breaks in the nucleus is Ku [a complex of Ku70 (XRCC6) and Ku80 (XRCC5) proteins]; it forms Ku-DNA complexes that serve as a structure binding other proteins. The next protein involved is DNA-PKcs, which binds to Ku-DNA and forms the DNA-PK complex, a nuclear serine/threonine protein kinase. Another enzyme associated with DNA-PKcs is a nuclease called Artemis with both endo- and exonuclease activities. Polymerases mu and lambda, members of the Pol X family, synthesize missing DNA nucleotides and the XRCC4-DNA ligase IV complex ligates DNA strands.

In the present study we investigated the levels of markers of oxidative damage to DNA (8-oxodG), lipids (15-F_{2t}-isoprostane, 15-F2t-IsoP) and proteins (protein carbonyl groups), chromosomal aberrations (F_G/100, %AB.C., ace) and the expression of selected genes participating in BER (including *OGG1*, *APEX1*, *XRCC1*) and NHEJ (including *LIG4*, *XRCC4*, *XRCC5* and *XRCC6*) in 64 subjects living and working in Prague and 75 subjects from the heavily polluted city of Ostrava. The genes were selected based on the fact that they participate in the repair of 8-oxodG and affect the frequency of chromosomal aberrations. Our study is a part of a large molecular epidemiological study that investigates the effect of air pollution on human health in two regions of the Czech Republic, Prague and Ostrava. The Ostrava region is currently the most polluted part of the country. We hypothesized that due to the air pollution, the subjects from Ostrava would exhibit significantly higher levels of oxidative damage to macromolecules, as well as an elevated frequency of chromosomal aberrations. We also expected the expression of selected genes to be upregulated in subjects from the Ostrava region. Our study is unique because it measures for the first time the levels of biomarkers in subjects exposed to air pollutants in concentrations that are unprecedented in the entire European Union. In the past we analyzed the frequency of chromosomal aberrations in policemen from Prague [16–19] and the levels of oxidative stress markers in Prague's bus drivers [20–22] and policemen. However, the present study is our first in which the assessment of oxidative stress markers and chromosomal aberrations is accompanied by the measurement of DNA repair gene expression.

2. Materials and methods

2.1. Subjects and sampling

The study population consisted of 64 city policemen working in Prague, Czech Republic, and 75 office workers working in Ostrava, Czech Republic. The study subjects were followed in the winter season of 2010. Each participant completed a questionnaire on their personal medical history and lifestyle. All participants signed an informed consent form and could cancel their participation at any time during the study, according to the Helsinki II declaration. The study was approved by the Ethical Committee of the Institute of Experimental Medicine AS CR in Prague. Any person who underwent medical treatment, radiography or vaccination up to 3 months before sampling was not included in the study.

From each subject, a spot urine sample was obtained. The blood samples were collected by venipuncture into vacuettes containing sodium heparin (for cytogenetic analysis) or EDTA (for oxidative stress markers and gene expression analysis). Samples were coded, transported to the Laboratory of Genetic Ecotoxicology and processed. Samples to be analyzed for levels of oxidative stress markers were kept in aliquots at −80 °C.

2.2. Exposure assessment

The subjects' exposure to c-PAHs was monitored by personal samplers during 48 h. The samplers were equipped with filters collecting particles of aerometric diameter ≤ 2.5 μm (PM2.5) [23]. Quantitative chemical analysis of c-PAHs (benz[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, dibenzo[a,h]anthracene, benzo[g,h,i]perylene and indeno[1,2,3-cd]pyrene) was performed by HPLC with fluorescence detection according to the EPA method [24] in the certified laboratory ALS Czech Republic, Prague.

Benzene was collected on Radiello® radial diffusive samplers (Supelco, Park Bellefonte, PA, USA) worn by study subjects for 24 h. It was adsorbed on graphitized charcoal and recovered by thermal desorption. The analysis was performed by capillary gas chromatography with flame ionization technique detection in the certified laboratory ALS Czech Republic, Prague.

2.3. Analysis of oxidative stress markers

8-OxodG ELISA. Urinary 8-oxodG levels were analyzed by competitive ELISA essentially as previously described [25,20]. Urinary 8-oxodG concentration was expressed as nmol 8-oxodG/mmol creatinine.

15-F2t-IsoP ELISA. Plasma 15-F2t-IsoP levels were analyzed using immunoassay kits from Cayman Chemical Company (Ann Arbor, MI, USA) as previously described [26]. The 15-F2t-IsoP concentrations were expressed as pg 15-F2t-IsoP/ml plasma.

Protein carbonyl assay. The levels of protein carbonyl groups were assessed in blood plasma using a noncompetitive ELISA, as previously described [27], with some modifications [20,28]. Plasma protein carbonyl concentration was expressed as nmol carbonyl/ml plasma.

2.4. Fluorescence in situ hybridization (FISH)

Whole venous blood cultures were established within 24 h after blood collection, according to the method described by Rossner et al. [29]. The protocol used to perform FISH with whole chromosome probes for chromosomes #1 and #4 was described in detail by Beskid et al. [19].

2.5. Gene expression analysis

Sample processing. To separate the leukocytes from the whole blood samples and isolate RNA, the LeukoLOCK™ Total RNA Isolation System (Ambion Inc., Austin, TX, USA) was used. The cells were then stored at −20 °C for several weeks until RNA extraction was performed.

RNA extraction. The extraction of leukocyte RNA was conducted according to the manufacturer's recommendations. RNA was quantified spectrophotometrically using a Nanodrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The integrity of RNA was determined using an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA, USA). The vast majority of samples (85%) had a RNA integrity number (RIN) between 6.0 and 9.0. Only ten samples had a RIN lower than 5.0.

Reverse transcription. The Transcriptor High Fidelity cDNA synthesis Kit (ROCHE, Mannheim, Germany) was used. cDNA was produced starting with 0.5 or 1 μg of total RNA. The original protocol was modified by using 2.5 μM oligo$(dT)_{18}$ and 10 μM random hexamers for priming and setting the incubation time and temperature to 30 min and 50 °C, respectively. Total reaction volume was 20 μl.

Quantitative PCR (qPCR). For all qPCR measurements, the 7900HT Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA) was used. Each qPCR reaction was carried out in a final volume of 14 μl containing 3.5 μl of diluted cDNA, 2.8 μl of water and 7 μl of TaqMan 2× Universal PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA). For determining the expression of the genes *APEX1*, *GAPDH*, *OGG1*, *XRCC1*, *XRCC4*, *XRCC5* and *XRCC6*, the reaction mixture contained an additional 200 nM forward and 200 nM reverse primers and 100 nM probe (Universal ProbeLi-

brary; Roche, Mannheim, Germany). The expression of *ACTB* and *LIG4* was assessed using 0.7 μl of RealTime Ready assays per reaction. Cycling conditions were: 10 min at 95 °C followed by 40 cycles of amplification (15 s at 95 °C and 60 s at 60 °C).

Data analysis. Raw data were analyzed with SDS Relative Quantification Software version 2.3 (Applied Biosystems, USA) to assign the baseline and threshold for Cq determination. Cq values were further analyzed using GenEx software version 5.2.7 (MultiD Analyses AB, Goteborg, Sweden). The expression levels of the target genes were normalized to the expression levels of the reference genes *GAPDH* and *ACTB*.

Further details about the gene expression analysis are given in the Supplementary material.

2.6. Plasma lipids, vitamins and cotinine assay

The plasma levels of cholesterol, LDL- and HDL-cholesterol and triglycerides were determined spectrophotometrically using diagnostic kits (BioVendor, Brno, Czech Republic) and appropriate standards. Levels of vitamins A, E and C in plasma were analyzed by HPLC [30,31]. Urinary cotinine levels as a marker of exposure to tobacco smoke were analyzed by radioimmunoassay [32].

2.7. Statistical analysis

We first compared the basic characteristics of the study groups (age, exposure to air pollutants, levels of cotinine, plasma lipids and vitamins) and the levels of biomarkers of oxidative stress, cytogenetic parameters and gene expression data. We used the Mann–Whitney *U*-test for those variables that did not follow a normal distribution and the *t*-test for data distributed normally. We further transformed the investigated variables into a two-level scale using medians and analyzed by multivariate logistic regression associations between: (a) oxidative stress markers and age, sampling location, exposure to benzene and environmental tobacco smoke (ETS; cotinine levels), plasma lipids and vitamins, and genes participating in BER; (b) cytogenetic parameters and age, sampling location, benzene and ETS exposure, plasma lipids and vitamins and genes participating in NHEJ. Exposure to c-PAHs, B[a]P and education level were found to be collinear with the sampling location and therefore these parameters were omitted in multivariate models. Using logistic regression we further analyzed the association between BER genes and vitamin levels and sampling locations and the expression of BER and NHEJ genes.

To correct for multiple comparisons, we used the FDR method (QVALUE software [33]). All other analyses were performed using SPSS 19.0 software.

3. Results

The basic characteristics of the study population are presented in Table 1. The age of the subjects was the same in both locations ($p = 0.338$). The exposure to air pollutants measured by personal monitors was significantly lower in subjects from Prague than in subjects from Ostrava (mean values ± SD: benzene: 5.64 ± 1.68 vs. 17.81 ± 11.78 μg/m^3, $p < 0.001$; B[a]P: 2.81 ± 1.88 vs. 17.09 ± 16.85 ng/m^3, $p < 0.001$; c-PAHs: 19.60 ± 14.56 vs. 118.7 ± 117.2 ng/m^3, $p < 0.001$ for subjects from Prague and Ostrava, respectively). Subjects from Prague were exposed to higher levels of ETS than subjects from Ostrava (mean values of cotinine ± SD: 65.12 ± 223.3 vs. 8.44 ± 6.51 ng/mg creatinine for subjects from Prague and Ostrava, respectively). We further analyzed the parameters related to nutrition that may influence oxidative stress and/or cytogenetic markers: plasma lipids and vitamins A, C and E. Our results indicate that subjects from Ostrava had a healthier lifestyle. While the levels of triglycerides and LDL cholesterol were lower in the Ostrava subjects, HDL cholesterol levels were lower among the subjects from Prague (mean ± SD: triglycerides: 1.51 ± 0.83 vs. 1.24 ± 0.69 mmol/l, $p = 0.016$; LDL cholesterol: 2.64 ± 0.64 vs. 2.39 ± 0.51 mmol/l, $p = 0.010$; HDL cholesterol: 1.14 ± 0.27 vs. 1.35 ± 0.27 mmol/l, $p < 0.001$ for subjects from Prague and Ostrava, respectively). Vitamin levels were significantly higher in the subjects from Ostrava (mean ± SD: vitamin A: 0.80 ± 0.34 vs. 1.09 ± 0.42 mg/l, $p < 0.001$; vitamin C: 8.39 ± 3.15 vs. 11.85 ± 4.14 mg/l, $p < 0.001$; vitamin E: 9.83 ± 3.96 vs. 17.09 ± 8.81 mg/l, $p < 0.001$, for subjects from Prague and Ostrava, respectively).

Although the exposure to benzene, B[a]P and c-PAHs was substantially higher in the Ostrava region, the levels of the analyzed biomarkers did not correspond with this trend (Table 2). Oxidative damage to macromolecules, cytogenetic parameters, as well as the expression of selected BER and NHEJ genes did not differ between the two locations. The difference in the genomic frequency of translocations (F_G/100) was close to the borderline of significance ($p = 0.060$), but contrary to our expectations, the values were higher in subjects from Prague. The expression of *XRCC4* was also higher in subjects from Prague ($p = 0.038$). After correction for multiple comparisons, the difference was no longer statistically significant ($q = 0.152$).

The results of multivariate logistic regression analyses investigating associations between oxidative stress markers in all subjects and other studied parameters are reported in Table 3. Higher levels of plasma vitamins C and E protect the organism against oxidative DNA damage: subjects with above-median levels of 8-oxodG in the urine had significantly lower plasma levels of vitamin C (odds ratio (OR), 95% CI: 0.37, 0.16–0.83), $p = 0.016$) and E (OR, 95% CI: 0.25, 0.08–0.75, $p = 0.013$). Vitamin C further protects the organism against lipid peroxidation: subjects with above-median levels of 15-F2t-IsoP had lower vitamin C levels (OR, 95% CI: 0.45, 0.20–1.00, $p = 0.049$). Above-median levels of 8-oxodG were further associated with the elevated expression of *XRCC1* (OR, 95% CI: 2.53, 1.05–6.09, $q = 0.048$). Associations between 15-F2t-IsoP and protein carbonyls with the expression of BER genes were not calculated, because BER does not repair oxidatively damaged lipids and proteins. Unexpectedly, above-median levels of protein carbonyls were associated with elevated plasma concentrations of vitamin A (OR, 95% CI: 3.39, 1.25–9.16, $p = 0.016$). In Table 4 the associations between the cytogenetic parameters and other variables are shown. Both F_G/100 and the percentage of aberrant cells (%AB.C.) were positively associated with age (OR, 95% CI: 3.94, 1.78–8.71, $p = 0.001$; 4.09, 1.84–9.12, $p = 0.001$, for F_G/100 and %AB.C., respectively). Exposure to benzene was positively associated with %AB.C. (OR, 95% CI: 3.50, 1.12–10.97; $p = 0.031$). Contrary to our expectations, subjects from Ostrava were less likely to have above-median levels of %AB.C. (OR, 95% CI: 0.18, 0.05–0.67, $p = 0.010$). The expression levels of none of the selected

Table 1
Characteristics of the study groups.

Variable	Prague ($N = 64$)		Ostrava ($N = 75$)		p
	Mean ± SD	Median (min, max)	Mean ± SD	Median (min, max)	
Age (years)	39.0 ± 8.5	37.5 (25.1, 61.9)	38.3 ± 9.9	35.7 (26.7, 63.7)	0.338
Benzene (μg/m^3)	5.64 ± 1.68	5.49 (2.28, 10.80)	17.81 ± 11.78	12.90 (5.34, 49.7)	<0.001
B[a]P (ng/m^3)	2.81 ± 1.88	2.44 (0.3, 11.5)	17.09 ± 16.85	8.42 (2.2, 74.2)	<0.001
c-PAHs (ng/m^3)	19.60 ± 14.56	17.78 (2.14, 103.9)	118.7 ± 117.2	54.72 (14.74, 513)	<0.001
Cotinine (ng/mg creatinine)	65.12 ± 223.3	12.26 (4.33, 1272)	8.44 ± 6.51	6.67 (1.97, 38.8)	<0.001
Triglycerides (mmol/l)	1.51 ± 0.83	1.35 (0.53, 5.63)	1.24 ± 0.69	1.09 (0.43, 4.29)	0.016
Total cholesterol (mmol/l)	4.57 ± 0.99	4.49 (1.59, 8.28)	4.60 ± 0.71	4.50 (3.33, 6.39)	0.812
HDL cholesterol (mmol/l)	1.14 ± 0.27	1.11 (0.56, 1.94)	1.35 ± 0.27	1.34 (0.89, 2.26)	<0.001
LDL cholesterol (mmol/l)	2.64 ± 0.64	2.57 (0.86, 5.11)	2.39 ± 0.51	2.28 (1.64, 4.35)	0.010
Vitamin A (mg/l)	0.80 ± 0.34	0.80 (0.22, 1.80)	1.09 ± 0.42	1.09 (0.24, 2.30)	<0.001
Vitamin C (mg/l)	8.39 ± 3.15	8.30 (1.5, 21.7)	11.85 ± 4.14	11.80 (4.2, 22.6)	<0.001
Vitamin E (mg/l)	9.83 ± 3.96	9.90 (2.4, 19.0)	17.09 ± 8.81	16.80 (3.0, 39.1)	<0.001

Table 2
Parameters of oxidative stress, DNA damage and gene expression in study subjects.

Variable	Prague ($N = 64$)		Ostrava ($N = 75$)		p	q
	Mean ± SD	Median (min, max)	Mean ± SD	Median (min, max)		
8-oxodG (nmol/mmol creat.)	4.84 ± 1.61	4.99 (0.52, 9.87)	4.28 ± 2.27	4.16 (0.19, 10.44)	0.102	
15-F_{2t}-isoprostane (pg/ml)	257.0 ± 105.4	238.6 (119.6, 647.8)	282.5 ± 120.8	273.5 (90.6, 814.6)	0.192	
Protein carbonyls (nmol/ml)	23.6 ± 6.48	22.4 (10.0, 39.1)	22.6 ± 6.79	21.3 (11.3, 43.3)	0.348	
F_G/100	1.37 ± 1.03	1.12 (0, 4.11)	1.11 ± 1.09	0.75 (0, 5.23)	0.060	
%AB.C.	0.23 ± 0.15	0.20 (0, 0.60)	0.20 ± 0.16	0.12 (0, 0.70)	0.120	
Ace	0.29 ± 0.68	0 (0, 4.0)	0.29 ± 0.62	0 (0, 3.0)	0.701	
OGG1	1.04 ± 0.33	0.99 (0.44, 2.84)	1.04 ± 0.28	0.94 (0.57, 1.94)	0.923	0.923
APEX1	1.02 ± 0.31	0.97 (0.46, 2.22)	1.04 ± 0.24	1.00 (0.64, 1.75)	0.701	0.923
XRCC1	1.08 ± 0.36	1.09 (0.51, 1.96)	1.02 ± 0.29	0.92 (0.51, 1.92)	0.235	0.705
LIG4	1.06 ± 0.39	1.00 (0.40, 2.13)	1.05 ± 0.29	1.01 (0.47, 1.80)	0.925	0.925
XRCC4	1.09 ± 0.31	1.03 (0.39, 1.92)	0.99 ± 0.26	0.99 (0.49, 1.81)	0.038	0.152
XRCC5	0.99 ± 0.19	0.99 (0.67, 1.63)	1.04 ± 0.19	1.04 (0.63, 1.45)	0.084	0.168
XRCC6	1.03 ± 0.25	1.04 (0.50, 1.79)	1.02 ± 0.20	1.00 (0.61, 1.63)	0.641	0.855

q—adjusted for multiple comparisons.

Table 3
Associations of selected parameters with oxidative stress markers.

Variable	8-oxodG (OR[a] (95% CI, p; q))	15-F2t-IsoP (OR[a] (95% CI, p))	Carbonyl (OR[a] (95% CI, p))
Age (below/above median)	1.42 (0.64, 3.13), 0.384	0.58 (0.27, 1.27), 0.174	1.60 (0.76, 3.39), 0.217
Location (Prague/Ostrava)	0.49 (0.14, 1.68), 0.258	1.96 (0.59, 6.49), 0.271	0.52 (0.16, 1.68), 0.274
Benzene (below/above median)	1.80 (0.62, 5.18), 0.277	0.61 (0.23, 1.64), 0.329	12.13 (0.79, 5.70), 0.134
Cotinine (below/above median)	0.64 (0.27, 1.56), 0.327	1.51 (0.655 3.45), 0.337	1.06 (0.48, 2.36), 0.891
Total cholesterol (below/above median)	1.30 (0.42, 3.99), 0.650	2.54 (0.84, 7.61), 0.095	2.21 (0.77, 6.35), 0.141
Triglycerides (below/above median)	2.17 (0.87, 5.43), 0.098	0.96 (0.40, 2.29), 0.924	1.33 (0.57, 3.09), 0.506
HDL cholesterol (below/above median)	0.72 (0.27, 1.92), 0.512	1.99 (0.80, 4.98), 0.139	0.62 (0.25, 1.53), 0.297
LDL cholesterol (below/above median)	1.15 (0.36, 3.65), 0.808	0.82 (0.26, 2.55), 0.731	0.52 (0.17, 1.56), 0.244
Vitamin C (below/above median)	0.37 (0.16, 0.83), 0.016	0.45 (0.20, 1.00), 0.049	0.86 (0.40, 1.84), 0.693
Vitamin A (below/above median)	2.16 (0.76,6.18), 0.150	1.25 (0.46, 3.37), 0.664	3.39 (1.25, 9.16), 0.016
Vitamin E (below/above median)	0.25 (0.08, 0.75), 0.013	1.46 (0.53, 3.99), 0.463	0.71 (0.26, 1.93), 0.496
APEX (below/above median)	0.46 (0.16, 1.34), 0.155; 0.097	N/A	N/A
OGG1 (below/above median)	1.13 (0.39, 3.29), 0.826; 0.344	N/A	N/A
XRCC1 (below/above median)	2.53 (1.05, 6.09), 0.038; 0.048	N/A	N/A

q—adjusted for multiple comparisons; N/A—not applicable.
[a] Adjusted for all variables in the table.

NHEJ genes showed any association with the levels of F_G/100. For %AB.C. and ace, these associations were not calculated because NHEJ does not participate in their formation and/or repair.

Since vitamins C and E seem to prevent oxidative DNA damage and the levels of both vitamins were elevated in subjects from Ostrava, we investigated the associations between the expression of the BER genes and vitamin levels. The results of multivariate logistic regression are reported in Supplementary Table 3. We observed a significant increase in the expression of *XRCC1* in subjects with above-median levels of vitamin C (OR, 95% CI: 2.25, 1.00–5.02, $p = 0.048$).

Univariate and multivariate analyses of gene expression levels in both studied regions are reported in Table 5. The table summarizes the odds of expression of BER and NHEJ genes in subjects from Prague/Ostrava to be above/below median values. *XRCC5* was found to have above-median expression in subjects in the more polluted Ostrava region (OR, 95% CI: 3.33, 1.03–10.8, $q = 0.046$). The expression of other NHEJ or BER genes was not significantly changed.

Table 4
Associations of selected parameters with cytogenetic biomarkers.

Variable	F_G/100 (OR[a] (95% CI, p; q))	%AB.C. (OR[a] (95% CI, p))	ace (OR[a] (95% CI, p))
Age (below/above median)	3.94 (1.78, 8.71), 0.001	4.09 (1.84, 9.12), 0.001	1.03 (0.43, 2.48), 0.954
Location (Prague/Ostrava)	0.54 (0.16, 1.82), 0.319	0.18 (0.05, 0.67), 0.010	2.32 (0.52, 10.34), 0.269
Benzene (below/above median)	2.56 (0.90, 7.34), 0.079	3.50 (1.12, 10.97), 0.031	1.07 (0.33, 3.52), 0.906
Cotinine (below/above median)	1.31 (0.57,2.99), 0.525	1.55 (0.66, 3.64), 0.316	1.89 (0.73, 4.88), 0.190
Total cholesterol (below/above median)	0.84 (0.28, 2.53), 0.757	2.21 (0.72, 6.80), 0.168	0.86 (0.24, 3.09), 0.819
Triglycerides (below/above median)	0.93 (0.39, 2.22), 0.878	1.01 (0.41, 2.51), 0.978	1.96 (0.71, 5.44), 0.196
HDL cholesterol (below/above median)	0.70 (0.27, 1.82), 0.467	0.91 (0.35, 2.40), 0.845	1.81 (0.65, 5.07), 0.259
LDL cholesterol (below/above median)	1.90 (0.62, 5.81), 0.261	0.83 (0.26, 2.62), 0.749	1.01 (0.28, 3.72), 0.987
Vitamin C (below/above median)	1.37 (0.62, 3.05), 0.440	1.65 (0.73, 3.75), 0.233	0.46 (0.18, 1.15), 0.097
Vitamin A (below/above median)	1.41 (0.50, 4.03), 0.519	0.94 (0.33, 2.70), 0.913	0.34 (0.10, 1.12), 0.076
Vitamin E (below/above median)	0.73 (0.25, 2.14), 0.571	1.02 (0.35, 2.98), 0.975	1.67 (0.51, 5.49), 0.397
LIG4 (below/above median)	0.87 (0.37, 2.01), 0.735; 0.980	N/A	N/A
XRCC4 (below/above median)	1.01 (0.47, 2.18), 0.980; 0.980	N/A	N/A
XRCC5 (below/above median)	0.58 (0.23, 1.42), 0.233; 0.932	N/A	N/A
XRCC6 (below/above median)	1.05 (0.46, 2.40), 0.917; 0.980	N/A	N/A

q—adjusted for multiple comparisons; N/A—not applicable.
[a] Adjusted for all variables in the table.

Table 5
Univariate and multivariate estimates of associations between gene expression and sampling locations.

Variable	Univariate analysis			Multivariate analysis		
(Prague/Ostrava)	Crude OR (95% CI)	*p*	*q*	OR (95% CI)	*p*	*q*
OGG1	0.65 (0.33, 1.26)	0.200	0.127	0.35[a] (0.11, 1.12)	0.077	0.171
APEX	1.15 (0.59, 2.25)	0.675	0.285	0.74[a] (0.24, 2.30)	0.603	0.504
XRCC1	0.57 (0.29, 1.13)	0.106	0.127	0.79[a] (0.25, 2.46)	0.680	0.504
LIG4	1.15 (0.59, 2.25)	0.675	0.165	1.35[b] (0.44, 4.16)	0.606	0.170
XRCC4	0.92 (0.47, 1.78)	0.793	0.165	0.78[b] (0.26,2.40)	0.670	0.170
XRCC5	2.07 (1.05, 4.09)	0.035	0.029	3.33[b] (1.03,10.8)	0.045	0.046
XRCC6	0.73 (0.37, 1.42)	0.346	0.144	0.74[b] (0.24, 2.28)	0.604	0.170

q—Adjusted for multiple comparisons.
[a] Adjusted for location, exposure to benzene, levels of cotinine, total cholesterol, triglycerides, HDL cholesterol, LDL cholesterol, vitamin C, A, E, age, 8-oxodG.
[b] Adjusted for location, exposure to benzene, levels of cotinine, total cholesterol, triglycerides, HDL cholesterol, LDL cholesterol, vitamin C, A, E, age, F_G/100.

4. Discussion

In the present study we investigated the levels of markers of oxidative stress, cytogenetic parameters and the expression of selected genes participating in DNA repair pathways in subjects living in the heavily polluted city of Ostrava and compared them with subjects living in a relatively clean location, the city of Prague. Despite high levels of air pollution in Ostrava, no analyzed biomarker was elevated in subjects from this region. However, our results suggest that other factors, namely gene expression and lifestyle factors, may compensate for high levels of air pollution.

In our previous studies we analyzed oxidative damage in environmentally exposed populations: in bus drivers [21,22] and city policemen [18] from Prague. In these studies oxidative damage to DNA and proteins was not elevated in subjects exposed to above-median levels of c-PAHs, B[a]P and/or benzene. Only lipid peroxidation was significantly higher in subjects of the bus drivers study exposed to above-median levels of c-PAHs and B[a]P. Subjects exposed to above-median benzene concentrations had non-significantly increased lipid peroxidation levels. In the study of city policemen, lipid peroxidation was non-significantly elevated in the winter sampling period when the ambient air was more polluted. There is, however, a substantial difference between the above-mentioned results and the present data. In the previous studies the levels of air pollution were significantly lower (mean B[a]P concentrations ranged from 0.18 to 2.12 ng/m^3), while the actual levels of B[a]P in Ostrava reached up to 74.2 ng/m^3. Due to these extreme concentrations of air pollutants, we expected to observe differences in the levels of the analyzed biomarkers between the two locations. Another reason for our assumption was that other authors have reported increased oxidative stress after environmental [34–36] and occupational [37–40] exposure to PAHs. Similarly, exposure to benzene was found to be associated with oxidative stress by some authors [41,42].

It is well known that vitamins, particularly vitamins C and E, act as antioxidants in the organism. The ability of vitamin A to scavenge free radicals seems to be limited [43]. In a number of studies the effect of vitamin C alone, or vitamin C supplemented with vitamin E and/or other vitamins on oxidative damage in the DNA of blood cells has been studied (reviewed in [44]). In most, but not all of these studies, the levels of 8-oxodG or other measured markers of oxidative stress decreased after supplementation of the study subjects with vitamins. Urinary excretion of 8-oxodG was also found to be negatively correlated with plasma vitamin C levels [45]. In our study the plasma levels of vitamins A, C and E were significantly higher in subjects living in the more polluted Ostrava region. We observed a negative association between the levels of vitamins C and E and the urinary excretion of 8-oxodG. We hypothesize that the antioxidant properties of both vitamins decreased the formation of 8-oxodG in cellular DNA and thus the urinary excretion of the oxidized base.

Another aspect that may play a role in the unexpectedly low levels of oxidative stress biomarkers in the Ostrava group is the effect of vitamins C and E on the expression of various genes that may influence the studied biomarkers. Vitamin C serves as a molecule affecting cellular stress responses, cell differentiation, proliferation, cell death and DNA repair [44,46]. There is even a report showing that vitamin C supplementation results in increased concentrations of OGG1 mRNA and a decrease in 8-oxodG levels in lymphocyte DNA [47]. Vitamin E modulates the expression of a number of genes that belong to two signal transduction pathways: a pathway centered on protein kinase C and a pathway centered on phosphatidylinositol 3-kinase [48]. We may assume that the elevated vitamin C and E levels in the Ostrava group upregulated certain genes that affected repair mechanisms, resulting in lower than expected urinary 8-oxodG concentrations. Our data indicate that *XRCC1* may be one of these genes. XRCC1 is a scaffold protein that interacts with most components of the short patch BER pathway [49]. It is essential for the coordination of all BER steps. It has been shown that the interaction of XRCC1 with OGG1 results in a 2- to 3-fold stimulation of the DNA glycosylase activity of OGG1. XRCC1 also passes on the DNA intermediate from OGG1 to APEX1, which leads to the acceleration of BER [50]. Thus, we can speculate that elevated *XRCC1* expression associated with above-median levels of vitamin C results in better orchestration of BER, which in turn leads to the faster removal of 8-oxodG from DNA and a higher repair efficiency. The fact that we collected spot urine samples may explain why we did not see any increase in 8-oxodG levels in the Ostrava subjects: due to the faster and more efficient DNA repair, some (a substantial amount of) 8-oxodG was already excreted from the body before the collection of the urine.

We have already analyzed chromosomal aberrations by FISH in several environmentally exposed populations, but none from a heavily polluted region comparable with Ostrava [16,18,19,51]. The frequency of chromosomal aberrations was affected by air pollution in groups exposed to B[a]P concentrations of at least 1.0 ng/m^3. Thus, DNA damage was elevated in subjects exposed to an average B[a]P concentration of 1.6 ng/m^3 vs. those exposed to 0.4 ng/m^3 B[a]P [16], in city policemen exposed to 1.6 ng/m^3 vs. controls exposed to 0.8 ng/m^3 B[a]P [51] and in subjects exposed up to 3.8 ng/m^3 B[a]P vs. controls exposed to an average B[a]P concentration up to 2.0 ng/m^3 [19]. It is noteworthy that in the last study, no linear dose-response relationship between F_G/100 and exposure to B[a]P was observed for B[a]P concentrations above 3.6 ng/m^3. On the other hand, no effect of environmental pollution on F_G/100 was observed in city policemen with an average B[a]P exposure of 1.0 ng/m^3 vs. those exposed to 0.2 ng/m^3 [18]. Our present data show that despite extreme exposure to B[a]P in Ostrava, the subjects from this region had comparable or lower levels of transloca-

tions than subjects from Prague. No difference in the levels of acentric fragments between the two locations was observed. Further, our results indicate that changes in the gene expression of *XRCC5* in the Ostrava subjects may be one of the factors responsible for this paradoxical observation. Other factors not analyzed in the present study may include the effectiveness of mRNA translation and the subsequent formation of DNA repair proteins and their post-translational modifications. Currently, we have no explanation as to why only the expression of *XRCC5* mRNA, but not other studied DNA repair genes, was upregulated in the subjects from Ostrava.

NHEJ is one of the pathways repairing DNA double strand breaks (DSB). The repair may be carried out directly by re-ligation of strand breaks; in this case the repair is precise, and no errors are introduced into the DNA strands. If, however, the sequences surrounding the break are lost, a part of the DNA sequence is deleted, the repair is not error-free and chromosomal translocations may be induced [52]. XRCC5 (Ku80), together with XRCC6 (Ku70) form a heterodimer complex Ku, which is the first protein recognizing DSBs and binding to the ends of DNA. Ku is abundant in cells; it is estimated that the average distance between two Ku molecules is only 4–6 times the Ku diameter [53]. Thus, early detection of DSB is probably not a factor limiting the efficiency of NHEJ [53]. Surprisingly, according to an *in vitro* study in rat cells, overexpression of the human Ku complex leads to the reduced repair capacity of DSB [54]. Although we are well aware of the limitations of comparisons between *in vitro* and *in vivo* conditions, this observation may partly explain our unexpected results: the elevated expression of *XRCC5* reduces the activity of the NHEJ pathway, which in turn results in a lower than expected frequency of chromosomal aberrations. Another explanation is related to the mechanism of the precise NHEJ. It has been shown that BRCA1 protein participates in the precise end-joining by suppressing the activity of the MRN complex, a complex of the proteins RAD50/MRE11/NBS1 that removes sequences flanking a DSB and allows NHEJ to repair the break by an error-prone mechanism [55]. We may speculate that in the Ostrava subjects the precise end-joining mechanism was preferentially activated leading to DNA repair without the induction of chromosomal aberrations. These hypotheses, however, do not explain why there were no differences observed between the two locations for acentric fragments. It is possible that better vitamin supplementation, supposedly leading to higher ROS scavenging, may result in the lower than expected ace frequency in the Ostrava group. This statement is further supported by the fact that in our study, lipid peroxidation was non-significantly ($p = 0.068$, data not shown) associated with the frequency of ace.

5. Conclusions

Subjects living in the heavily polluted Ostrava region had comparable levels of oxidative stress markers and cytogenetic parameters as subjects from the substantially cleaner city of Prague. Our results suggest that the elevated expression of *XRCC5* and higher levels of vitamins C and E in the Ostrava group compensate for the negative effect of air pollution on human health.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mrfmmm.2011.06.001.

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Příloha 6:

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The impact of air pollution on the level of micronuclei measured by automated image analysis

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The impact of air pollution on the levels of micronuclei measured by automated image analysis

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ABSTRACT

The measurement of micronuclei (MN) in human peripheral blood lymphocytes is frequently used in molecular epidemiology as one of the preferred methods for assessing chromosomal damage resulting from environmental mutagen exposure. In the present study, we evaluated the effect of exposure to carcinogenic polycyclic aromatic hydrocarbons (c-PAHs), volatile organic compounds (VOC) and smoking on the frequency of MN in a group of 56 city policemen living and working in Prague. The average age of the participants was 34 ± 6 years. The study was conducted on the same subjects in February and May 2007. The concentrations of air pollutants were obtained from personal and stationary monitoring. A statistically significant decrease in the levels of pollutants was observed in May when compared with February, with the exception of toluene levels measured by stationary monitoring. The frequency of MN was determined by the automatic image scoring (MetaSystems Metafer 4, version 3.2.1) of DAPI-stained slides. The results of the image analysis indicated a significant difference in the frequency of MN (mean levels 7.32 ± 3.42 and 4.67 ± 2.92, for February and May, respectively). Our study suggests that automatic image analysis of MN is a highly sensitive method for evaluating the effect of c-PAHs and confirms that there are no differences between smokers and nonsmokers. These results demonstrate the ability of c-PAHs to increase MN frequency, even if the exposure to c-PAHs occurred up to 60 days before the collection of biological material. Our work is the first human biomonitoring study focused on the measurement of MN by automated image analysis for assessing chromosomal damage as a result of environmental mutagen exposure.

1. Introduction

Carcinogenic polycyclic aromatic hydrocarbons (c-PAHs) are among the most studied environmental pollutants, and their deleterious effect on human health has been shown repeatedly [1]. Exposure to c-PAHs is associated with numerous biological consequences, including increased levels of chromosomal aberrations [2,3].

The measurement of micronuclei (MN) in human peripheral blood lymphocytes is one of the methods frequently used in molecular epidemiology. MN represent a measure of both chromosome breakage and chromosome loss. Therefore, an increased frequency of micronucleated cells, used as a biomarker of genotoxic effects, can reflect exposure to agents with clastogenic or aneugenic modes of action [4]. Currently, the MN assay is one of the preferred methods for assessing chromosomal damage as a result of environmental mutagen exposure as well as a tool for genotoxicity testing.

The first attempt to use a micronucleus test was made in 1959 [5], when the first application for evaluating chromosomal aberrations was introduced. A shift to the cultivation of human peripheral lymphocytes [6] was a milestone in the development and future routine use of the method. Over the years, the method has been used for investigating genetic damage under different conditions. Some studies have been performed in vivo and some in vitro, and different stainings have been used. Currently, the measurement of MN in peripheral blood lymphocytes is the preferred approach, but epithelial cells, erythrocytes and fibroblasts have been used in many human biomonitoring studies as well.

The new assay procedure dates its origin to 1985, when cytochalasin-B was first used to inhibit cytokinesis [4]. A detailed description of an in vitro micronucleus technique that enables the measurement of chromosome loss and chromosome breakage was developed [7]. The cytokinesis-block micronucleus assay (CBMN) was later described as a "cytome" assay. This concept implies that

Abbreviations: B[a]P, benzo[a]pyrene; BNC, binucleated cell; CBMN assay, cytokinesis-block micronucleus assay; c-PAHs, carcinogenic polycyclic aromatic hydrocarbons; DAPI, 4′-6-diamidino-2-phenylindole; FISH, fluorescent in situ hybridization; HPLC, high performance liquid chromatography; HUMN, HUman MicroNucleus project; MN, micronuclei; PM10, particulate matter <10 μm; PM2.5, particulate matter <2.5 μm; ROI, region of interest; VAPS, versatile air pollution sampler; VOC, volatile organic compounds.

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every cell studied in the system is scored for its viability status, mitotic status, and chromosomal instability or damage status [8].

The determination of MN frequency by visual scoring has been used in many studies. Different approaches to the staining and scoring of cells as well as interpersonal differences have a strong impact on the interpretation of the results. The human micronucleus project (HUMN), established in 1997, became an important unifying factor for MN analysis [9]. A detailed description of the scoring criteria for lymphocytes [10] and an assessment of the sources of variability in the CBMN assay were achieved [11]. Analysis of the large data set of this project allowed researchers to achieve greater statistical power in the results. This analysis confirmed that MN frequency was not increased in moderate smokers; only heavy smokers (30 cigarettes or more per day) showed a significant increase in genotoxic damage as measured by the micronucleus assay [12]. The results from more than 6700 manually analyzed subjects confirmed that an elevated MN frequency is predictive of an increased cancer risk [13], similarly as was observed for chromosomal aberrations [14]. Though the visual scoring of micronuclei is relatively easy, the scoring of thousands of cells is very time-consuming. Owing to this fact, efforts to automate the counting of cells have been made. Hutter and Stöhr reported the automation of micronucleus analysis by flow cytometry as early as 1982 [15]. To obtain objective data quickly, an automatic technique with computerized image analysis was developed [16]. However, this system, allowing the automated detection of binucleated lymphocytes with or without MN, was capable of detecting only 63% of all binucleated cells (BNC) and 57% of all MN. This fact might be one of the reasons why visual scoring continued to predominate. The next comparison of visual and automated detection was done by Castelain et al. [17], who found a 59–86% effectiveness of BNC classification; the authors described problems with the detection of extremely small MN and the aggregation of MN to the main nucleus. Bocker et al. [18,19] published a similar evaluation of the automated scoring of MN in BNC.

The development of computer software allowed the application of more advanced automated image analysis of micronuclei in cytochalasin-B-blocked human lymphocytes. Recently, two new systems have been developed. The first system was introduced by MetaSystems [20]. Detailed testing and a comparison between samples stained with Giemsa for visual counting and with DAPI for automated analysis were performed in a group of breast cancer patients and controls to evaluate the performance of the automated analysis [21]. The results of this comparison indicated that the system appears to be more reliable for DAPI-stained samples using fluorescence. The second system was introduced in 2009 by Decordier et al. [22]. This system, suggested for biomonitoring, was developed for scoring Giemsa-stained slides.

Until now, automated image analysis of BNC has focused on validating the method in in vitro studies and on investigating chromosomal damage in cancer patients. Our study is the first biomonitoring study focused on the measurement of MN by automated image analysis for assessing chromosomal damage as a result of environmental mutagen exposure. We have already observed an increase in DNA adducts, and using fluorescent in situ hybridization (FISH), chromosomal aberrations during the winter in city policemen in Prague, when concentrations of c-PAHs determined by personal monitoring were 9.7 ng/m^3, benzo[a]pyrene (B[a]P) 1.6 ng/m^3 [2,23]. In our new project, we evaluated the effect of exposure to c-PAHs, VOC, smoking and genetic polymorphisms on genetic damage in somatic and gametic cells in a group of city policemen living and working in Prague. As biomarkers, we used DNA adducts, chromosomal aberrations and micronuclei in peripheral lymphocytes and DNA fragmentation in sperm. The study was conducted in February and May 2007 [24].

We tested the hypothesis, that the frequency of MN by automated image analysis of DAPI-stained slides is the sensitive method to detect environmental exposure to c-PAHs in the peripheral lymphocytes.

2. Materials and methods

2.1. Study subjects

The study was performed in Prague, the capital city of the Czech Republic. The study population was a group of 56 city policemen working in the center of the city and spending more than 8 h a day outdoors. The group contained 20 smokers and 36 nonsmokers; the average age of the policemen was 34 ± 6 years. Most of the subjects (49) were examined repeatedly, in February and May 2007.

All individuals were interviewed and completed a questionnaire on smoking habits, X-ray examinations, viral infections, medication, and alcohol consumption within 3 months preceding blood sampling. In addition, cotinine levels in urine were measured to objectify smoking status. All participants signed an informed consent form and could cancel their participation at any time during the study, according to the Helsinki II declaration. The ethical committee of the Institute of Experimental Medicine AS CR in Prague approved the study.

The blood and urine samples were collected at the end of a working shift. The blood was drawn by venipuncture into vacuettes containing sodium heparin, coded and transported to the laboratory. The urine samples were kept at −80 °C until cotinine analysis.

2.2. Inhalation exposure

The sampling was carried out in winter (February 14–21, 2007) and spring (May 23–31, 2007). Individual exposure to air pollutants was measured for 48 h before the collection of biological samples using personal sampling devices (URG Corp, USA). Pallflex glass fiber filters with collected particulate matter of aerometric diameter < 2.5 μm (PM2.5) were extracted by dichlormethane. Quantitative chemical analysis of c-PAHs (benz[a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[ghi]perylene, benzo[a]pyrene, chrysene, dibenz[ah]-anthracene and indeno[cd]pyrene) was performed by HPLC with fluorimetric detection according to the EPA method in the certified laboratory ALS Czech Republic, Prague (EN ISO CSN IEC 17025). Personal monitoring was supplemented with daily air pollution data from stationary monitors located in the downtown area of Prague–Smichov. During the sampling period, the particulate air pollution monitored by versatile air pollution samplers (VAPS) was as follows in February vs. May: PM10, 6.5–89.5 μg/m^3 vs. 25.7–34.2 μg/m^3; PM2.5, 5.6–76.7 μg/m^3 vs. 16.8–37.6 μg/m^3; c-PAHs, 9.9–39.7 ng/m^3 vs. 1.9–3.5 ng/m^3; and B[a]P, 1.3–5.3 ng/m^3 vs. 0.1–0.3 ng/m^3. In the winter (January–February) vs. the spring (April–May), PM10 ranged from 6.5 to 89.5 μg/m^3 vs. 35.3 to 62 μg/m^3; PM2.5, 5.6–76.7 μg/m^3 vs. 24.8–48.5 μg/m^3; c-PAHs, 3.3–43.8 ng/m^3 vs. 1.9–9.9 ng/m^3; and B[a]P, 0.3–6.0 ng/m^3 vs. 0.1–1.4 ng/m^3, respectively.

In addition, personal monitoring of traffic-related VOC (benzene, toluene, ethylbenzene, *m*-, *p*- and *o*-xylene) was performed using Radiello® radial diffusive samplers (Supelco, PA, USA) worn by the study subjects for 24 h. VOC were trapped on graphitized charcoal by adsorption and recovered by thermal desorption. The analysis was performed by capillary gas chromatography with flame ionization detection in the certified laboratory ALS Czech Republic, Prague. The sorbent cartridges were contained in carrier tubes at all times before and after each sampling. To detect possible secondary contamination, blank samples were used with every sampling group. The Czech Hydrometeorological Institute, Prague, performed stationary monitoring of VOC continually at the same locations as VAPS monitoring by gas chromatography with flame/photo ionization detection.

2.3. Micronucleus test

2.3.1. Lymphocytes culture

Whole venous blood cultures were established within 24 h after blood collection, according to the method described by Sorsa et al. [25]. The cultures were prepared in RPMI 1640 medium (Sevapharma) supplemented with 20% calf serum (Biochrom AG) and 1% phytohemagglutinin (Biochrom AG). Two duplicate cultures were set up from each sample and cultivated at 37 °C. Thereafter, the cultures were incubated for 44 h, when cytochalasin B (Sigma) was added to a final concentration of 5 μg/ml [4]. After 72 h of incubation, cultures were harvested by centrifugation, treated with a hypotonic solution of KCl (0.075 M) and fixed repeatedly with methanol/acetic acid.

2.3.2. Micronuclei automated image analysis

After processing of the cultures and preparation of slides, the slides were dried and stained by DAPI for automated image analysis.

The automated scanning system Metafer 4, Version 3.2.1, from MetaSystems (Atlussheim, Germany) with a motorized Axio Imager Z1 microscope (Carl Zeiss, Germany) was used [20] to scan the DAPI-stained slides. Scanning of BNC was performed using a 10× objective (final magnification 100×) and a DAPI filter; 1000 BNC per subject were analyzed. All automated findings with one or more MN were

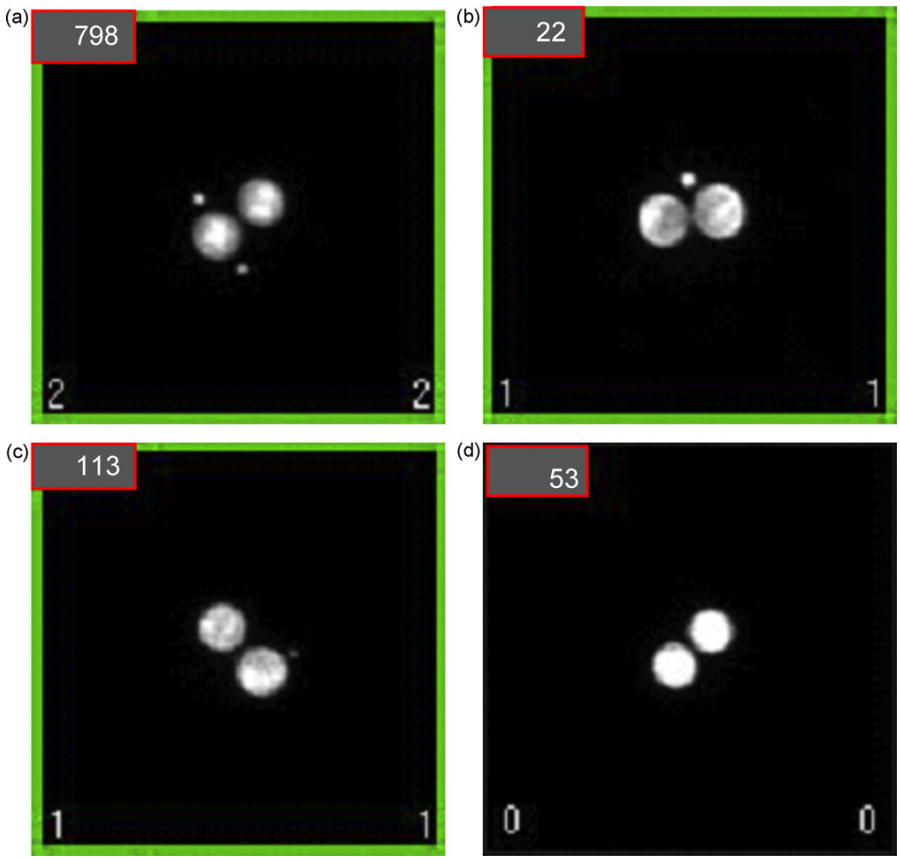

Fig. 1. Examples of cytochalasin-B-blocked BNC stained with DAPI from galleries of scanned slides. (a) BNC with two MN; (b) and (c) BNC with one MN of different sizes; (d) BNC without MN.

checked and corrected if necessary. One person scored all the slides, and after processing of all the images with MN, one additional check was performed. Examples of BNC with and without MN are shown in Fig. 1.

In this study, MicroNuc Classifier, Version 3.5.104, was used. The setting parameters used to identify binucleated cells and micronuclei were as follows:

Nuclei-image processing operations: sharpen (3,4); object threshold: 20%; minimum area: 4000 in 1/100 μm^2; maximum area: 20,000 in 1/100 μm^2; maximum relative concavity depth: 120 in 1/1000; maximum aspect ratio: 1500 in 1/1000; maximum distance: 180 in 1/10 μm; maximum area asymmetry: 90%; region of interest radius: 300 in 1/10 μm; maximum object area in the region of interest (ROI): 2000 in 1/100 μm^2.

Micronuclei-image processing operations: medianV (3), medianH (3), average (3,1), sharpen (5,5); object threshold: 15%; minimum area: 100 in 1/100 μm^2; maximum area: 2100 in 1/100 μm^2; maximum relative concavity depth: 1000 in 1/1000; maximum aspect ratio: 1720 in 1/1000; maximum distance: 290 in 1/10 μm.

2.4. Cotinine assay

Urinary cotinine levels, as the major nicotine metabolite, were analyzed by radioimmunoassay [26] to check the tobacco smoke exposure reported in the lifestyle questionnaires. Subjects with cotinine levels greater than 500 ng/mg of creatinine were considered active smokers.

2.5. Statistical analysis

Statistical analyses were performed using Statistica 7.1 (StatSoft, OK, USA) and SAS 9.1.3. (SAS Institute, NC, USA). Power analysis of the study revealed that to detect the decrease of MN frequency between February and May observed in our study, the required sample size is 28 (at 80% power and $p < 0.05$).

For data that were not distributed normally, the nonparametric Mann–Whitney Sum *U*-test was used for comparison of two groups or two sample periods. For subjects present in both periods, the Wilcoxon matched pairs test was used to confirm the difference in MN frequencies between February and May. Bivariate and multivariate logistic regression were performed to identify the impact of the monitored markers on the level of genetic damage. For logistic regression estimates, variables were transformed into a three level scale using tertiles or into a two level scale using medians. To assess the influence of pollutants on micronuclei frequencies, data from both seasons were pooled.

3. Results

A group of 56 city policemen, consisting of 20 smokers and 36 nonsmokers, was investigated. Forty-nine participants were investigated twice, in both sampling periods. The average age of all participants was 34 ± 6 years (35 ± 7 years for smokers and 33 ± 5 years for nonsmokers). Urinary cotinine levels were 1364 ± 1146 and 2336 ± 2006 ng/mg creatinine for smokers and 48 ± 205 and 23 ± 48 ng/mg creatinine for nonsmokers sampled in February 2007 and May 2007, respectively.

An overview of the mean values and standard deviations (SD) of c-PAH and VOC concentrations measured by personal and stationary monitoring in February 2007 and May 2007 is shown in Table 1. All monitored pollutants showed a decrease over the intervening period of 3 months, which is in agreement with long-term observations of pollutant levels in these seasons in the Czech Republic. Only the concentration of toluene measured by stationary monitoring did not differ between the two sampling periods. The decreases in the levels of c-PAHs, B[a]P, benzene and *o*-xylene measured by personal and stationary monitoring were statistically significant ($p < 0.05$–$p < 0.001$).

The mean values of the frequency of MN in both seasons for all participants and for those participants investigated twice are summarized in Table 2. Significantly higher levels of MN were detected in February than in May using the Mann–Whitney Sum *U*-test, as

Table 1
c-PAHs and VOC (Mean ± SD) measured by personal and stationary monitoring during February 2007 and May 2007.

Compound	Personal monitoring		Stationary monitoring	
	February	May	February	May
c-PAHs (ng/m^3)	6.15 ± 4.22**	2.17 ± 3.66	18.56 ± 11.19**	2.94 ± 0.86
B[a]P (ng/m^3)	1.04 ± 0.76**	0.24 ± 0.54	2.37 ± 1.47**	0.22 ± 0.16
VOC				
Benzene (μg/m^3)	6.99 ± 6.18**	4.53 ± 4.47	2.04 ± 0.81**	0.56 ± 0.26
Toluene (μg/m^3)	22.06 ± 67.59	13.99 ± 19.50	3.73 ± 1.54	4.07 ± 1.52
Ethylbenzene (μg/m^3)	5.86 ± 21.21	3.25 ± 4.58	0.53 ± 0.37**	0.21 ± 0.20
m-, *p*-Xylene (μg/m^3)	19.70 ± 76.84	10.95 ± 17.42	N/A	N/A
o-Xylene (μg/m^3)	6.90 ± 26.46*	3.75 ± 6.34	0.76 ± 0.53**	0.32 ± 0.25

February vs. May *$p < 0.05$, ** $p < 0.001$; N/A: data not available.

Table 2
MN frequencies (mean ± SD) per 1000 BNC in the group of policemen assessed by automated image analysis.

Samples group	N	MN/1000 BNC[a]	N	MN/1000 BNC[b]
February 2007				
All	56	7.32 ± 3.42**	49	7.16 ± 3.50**
Smokers	20	7.33 ± 2.92*	19	7.42 ± 3.02*
Nonsmokers	36	7.31 ± 3.72**	30	7.00 ± 3.81**
May 2007				
All	56	4.67 ± 2.92	49	4.37 ± 2.56
Smokers	20	4.77 ± 3.29	19	4.65 ± 3.42
Nonsmokers	36	4.61 ± 2.72	30	4.50 ± 2.52

N: number of subjects; February vs. May *$p < 0.01$, **$p < 0.001$.
[a] Mann–Whitney *U*-test.
[b] Wilcoxon matched pairs test

well as for paired samples using the Wilcoxon matched pairs test. No effect of smoking on MN frequency was observed.

In samples analyzed by automated image analysis, we found a significant association of MN frequencies with personal exposure to c-PAHs and *o*-xylene. The effect of exposure to B[a]P and ethylbenzene was on the borderline of significance. The odds ratios (OR) and 95% confidential intervals (CI) are reported in Table 3.

To evaluate the effect of c-PAH exposure over various periods of time on the frequency of MN, we used the data from stationary monitors that continuously measured the concentrations of c-PAHs in Prague. We then analyzed the association between MN frequency in the pooled data (112 subjects) and the concentrations of air pollutants during 3-, 7- and 15-day intervals measured up to 30 days before blood collection. The results of multivariate analysis for the 3-day period are reported in Table 4. The concentrations of c-PAHs and B[a]P were (with several exceptions) positively associated with the frequency of MN analyzed by the automated image system. The results for 15-day intervals measured up to 75 days before blood collection showed the same trend up to 60 days (Table 5). Thus, we may conclude that MN are already induced after a 3-day exposure to c-PAHs, and the same effect persists over the 60-day period before sample collection.

Table 3
Multivariate logistic regression analysis of environmental pollutants and their impact on the frequency of MN measured by automated image analysis.

	OR (95% CI)	*p*
Personal exposure–c-PAHs	2.01 (1.16–3.48)	<0.05
Personal exposure–B[a]P	2.42 (0.95–6.17)	0.07
Personal exposure–VOC		
Benzene	1.38 (0.83–2.32)	0.22
Toluene	0.83 (0.48–1.43)	0.50
Ethylbenzene	1.86 (1.00–3.43)	0.05
m-, *p*-Xylene	1.45 (0.84–2.51)	0.18
o-Xylene	2.02 (1.17–3.51)	<0.05

Table 4
Multivariate logistic regression of the frequency of MN measured by automated image analysis with c-PAHs measured by stationary monitoring over various 3-day periods before sample collection.

Compound	Period (days)	OR (95% CI)	p
c-PAHs	1–3	3.67 (1.78–7.59)	<0.001
	4–6	2.72 (1.50–5.03)	<0.01
	7–9	1.53 (0.90–2.61)	0.11
	10–12	1.36 (0.78–2.37)	0.27
	13–15	1.88 (1.09–3.26)	<0.05
	16–18	3.04 (1.60–5.80)	<0.001
	19–21	2.45 (1.27–4.73)	<0.01
	22–24	3.49 (1.78–6.86)	<0.001
	25–27	2.06 (1.15–3.69)	<0.05
	28–30	2.78 (1.54–5.03)	<0.001
B[a]P	1–3	3.67 (1.78–7.59)	<0.001
	4–6	2.72 (1.50–5.03)	<0.01
	7–9	1.53 (0.90–2.61)	0.11
	10–12	1.36 (0.78–2.37)	0.27
	13–15	1.88 (1.09–3.26)	<0.05
	16–18	2.56 (1.04–6.27)	<0.05
	19–21	1.92 (0.80–4.64)	0.15
	22–24	3.49 (1.78–6.86)	<0.001
	25–27	2.06 (1.15–3.69)	<0.05
	28–30	2.78 (1.54–5.03)	<0.001

Table 5
Bivariate and multivariate logistic regression of the frequency of MN measured by automated image analysis with c-PAHs measured by stationary monitoring over various 15-day periods before sample collection.

Period (days)	Bivariate logistic regression		Multivariate logistic regression	
	OR (95% CI)	*p*	OR (95% CI)	*p*
1–15	2.43 (1.40–4.22)	<0.01	2.26 (1.29–3.94)	<0.01
16–30	2.76 (1.55–4.91)	<0.001	3.04 (1.60–5.80)	<0.001
31–45	1.68 (1.00–2.82)	<0.05	1.64 (0.98–2.77)	0.06
46–60	2.76 (1.55–4.91)	<0.001	3.04 (1.60–5.80)	<0.001
61–75	1.45 (0.86–2.45)	0.16	1.31 (0.77–2.24)	0.32

4. Discussion

In the present study we analyzed the effect of air pollution and smoking on the frequency of MN by automated image analysis in a group of city policemen in two seasons with different air pollution levels.

Studies using automated MN scoring are scarce. To the best of our knowledge, only two automated systems have been tested recently [21,22]. Of these, only the study by Varga et al. [21] used the same system and MN staining as in our study. In Varga's study, the fluorescent dyes tested for automated image MN analysis were suggested to be more suitable than non-fluorescent dyes. Furthermore, DAPI staining was shown to minimize difficulties in MN counting due to cytoplasmatic staining. The results using DAPI-stained slides were slightly affected by the different settings of the classifier used for analysis. The classifier we used in our study, in combination

with DAPI staining, allows the detection of very small MN. The minimal threshold was set to one-sixteenth of the diameter of the main nuclei [10].

While MN frequencies have been analyzed in numerous studies, automated image scoring has been rarely used. According to a MEDLINE search, automated image studies have concentrated mostly on comparisons between healthy controls and subjects with various cancers (i.e. [27–29]). Therefore, studies with which we compared our results include those that used visual MN scoring, even though automated scoring would be more appropriate.

Our results indicate that MN frequencies, when measured by the automated scoring system, are significantly affected by higher levels of air pollutants. Others have published similar observations in general populations living in polluted regions [30,31] as well as in heavily exposed workers [32,33]. A recent meta-analysis of data from a 25-year period also indicates increased MN frequencies in environmentally exposed subjects, specifically children [34]. Our results also showed that MN frequency was affected by exposure to c-PAHs up to 60 days before sample collection. Concentrations of c-PAHs measured more than 60 days before the collection of samples had no effect on MN formation. Similar results were obtained using conventional cytogenetic analysis, where the frequency of aberrant cells corresponded to the exposure to chemical carcinogens during a period of 3 months [35].

Comparing stationary exposure in the center of Prague as well as the personal exposure of city policemen to c-PAHs in February 2007 vs. February 2001 [23] or January 2004 [3], exposure to c-PAHs in February 2007 was lower due to meteorological conditions, e.g. personal exposure to B[a]P was 1.04 ± 0.76 ng/m^3. It is important to note that even this B[a]P concentration increased the frequency of MN.

We did not find any significant difference between smokers and nonsmokers in either season using automated MN scoring. Based on a previous report [12], we could expect increased MN frequencies only in heavy smokers; such heavy smokers were not present among our study subjects.

VOC include a number of organic compounds, some of which are classified as human carcinogens (benzene, IARC, Group 1) [36], others as possible carcinogens (ethylbenzene, IARC, Group 2B) [37], while still others are not carcinogenic. We found a significant effect of *o*-xylene environmental exposure on the frequency of MN, while the effect of other compounds (*m*-xylene, *p*-xylene, ethylbenzene, benzene) was on the borderline of significance. The results reported by others have mainly focused on occupational exposure. A significant effect of VOC exposure on MN was found in petroleum refinery workers [38]. Previous studies that followed a group of service station attendants and aircraft maintenance personnel either did not see any association or else found inconsistent results [39,40]. A review by Duarte-Davidson et al. [41], focused on environmental exposure of the general population to benzene, concluded that there is no evidence yet to suggest that continuous exposure to environmental concentrations of benzene has adverse health effects. Our February concentrations were approximately 40% of the Czech standard for benzene environmental exposure.

We did not find any significant effect of age on MN frequency, while other studies have mostly shown an increase in MN frequency with age [42–44]. The negative results found in our study may be explained by the relatively narrow range in age of the studied subjects.

In conclusion, this work is the first human biomonitoring study focused on the measurement of MN by automated image analysis for assessing chromosomal damage as a result of environmental mutagen exposure. Our results demonstrate the ability of c-PAHs to increase MN frequency, even if the exposure to c-PAHs occurred up to 60 days before the collection of biological material. Further, our findings indicate the ability of the automated system to easily analyze slides with a low density of cells that would be very laborious to score using visual analysis. Considering the possibility to analyze more BNC more quickly, we suggest analyzing 2000–3000 BNC in future studies to obtain more statistically powerful data. Furthermore, we suggest that the classifier setting used in this work be used in other biomonitoring studies to allow for comparable inter-laboratory results.

Conflict of interest

None.

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Příloha 7:

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Automated scoring of lymphocyte micronuclei by the MetaSystems Metafer image cytometry system and its application in studies of human mutagen sensitivity and biodosimetry of genotoxin exposure

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REVIEW

Automated scoring of lymphocyte micronuclei by the MetaSystems Metafer image cytometry system and its application in studies of human mutagen sensitivity and biodosimetry of genotoxin exposure

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Automated image analysis scoring of micronuclei (MN) in cells can facilitate the objective and rapid measurement of genetic damage in mammalian and human cells. This approach was repeatedly developed and tested over the past two decades but none of the systems were sufficiently robust for routine analysis of MN until recently. New methodological, hardware and software developments have now allowed more advanced systems to become available. This mini-review presents the current stage of development and validation of the Metasystems Metafer MNScore system for automated image analysis scoring of MN in cytokinesis-blocked binucleated lymphocytes, which is the best-established method for studying MN formation in humans. The results and experience of users of this system from 2004 until today are reviewed in this paper. Significant achievements in the application of this method in research related to mutagen sensitivity phenotype in cancer risk, radiation biodosimetry and biomonitoring studies of air pollution (enriched by new data) are described. Advantages as well as limitations of automated image analysis in comparison with traditional visual analysis are discussed. The current increased use of the Metasystems Metafer MNScore system in various studies and the growing number of publications based on automated image analysis scoring of MN is promising for the ongoing and future application of this approach.

Introduction

Significant achievements

Historic steps to automation. The micronucleus (MN) assay in human peripheral lymphocytes is a frequently used method to assess chromosomal damage. Though the visual scoring of MN is relatively easy for a trained person, the scoring of thousands of cells is very time-consuming and tiring work. It may also result in subjective interpretation of scoring criteria (1). Automation by image analysis was presented in a review by Tates *et al.* (2) where the capacity of detection of binucleated cells (BNC) and MN was mentioned. These parameters were also mentioned in other studies (3–6), where automated systems were tested. Some limitations of these early systems were based on problems with the detection of (i) BNC in the cytokinesis-block MN assay, (ii) small MN and (iii) identifying MN very close to the nucleus (3). The cytokinesis-block MN assay has become the preferred method for scoring MN that are only expressed in cells which complete nuclear division after genotoxin exposure (7,8) and for this reason, automated image analysis scoring systems for MN are now based on identifying and scoring MN in BNC.

Besides other recent systems based on flow cytometry (9) or laser scanning cytometry (10), the development of improved computer software allowed the application of advanced image analysis systems for use in the cytokinesis-block MN test in human lymphocytes: Metafer MNScore (MetaSystems, GmbH Altlussheim, Germany) (11,12), a subject of this publication (Figure 1), was the first system developed; it was followed by PathFinder™Cellscan™ (IMSTAR, Paris, France) (13) described by Decordier *et al.* (14) in this issue. The Metafer system was introduced by MetaSystems in 2004 (11). The authors described new developments in automated cytogenetic imaging, including the unattended scoring of MN, automated scoring of dicentric chromosomes, the analysis of single-cell gel electrophoresis and the detection of fluorescent foci. Technical aspects and principles, including automated focusing, the motorised scanning stage with a capacity of eight slides, the external slide feeder for up to 80 slides and other details are presented.

In the same year when the Metafer MNScore system was presented, the results of a comparison between samples stained with Giemsa for visual scoring and with DAPI (4′,6-diamidino-2-phenylindole) for automated image analysis was published (12). The results of this comparison indicated that the system appeared to be highly reliable for DAPI-stained samples using fluorescence (besides other fluorescent dyes such as Hoechst 33529, ethidium bromide or propidium iodide). Another study, however, presented an application for analysis in transmitted light using non-fluorescent dyes, which allows staining of both, chromatin and cytoplasm (15). In this study, May-Grűnwald–Giemsa staining was used, but the authors concluded that use of fluorescent dyes may lower the number of a false-positive count.

The procedure of automated image analysis. Metafer MNScore separately identifies mononucleated cells or BNC and MN in the vicinity of these cells. The parameters for identification of both is stored in a set of parameters (called a classifier).

BNC are detected by identification of two nuclei matching morphology criteria given in the classifier (size and shape). Subsequently, the distance of the nuclei and their size ratio is analysed. Finally, a region of interest around the nuclei is analysed for other objects, and the nuclei are rejected from analysis, if the area of these objects exceeds a value defined in the classifier. The latter is used to avoid that other nuclei nearby are identified as false-positive MN.

MN in the region of interest are indentified based on morphology criteria given in the classifier.

It is possible to apply image-processing algorithms to the images, separately for the identification of nuclei and of MN. In order to increase the number of detected nuclei, an automated separation algorithm for the separation of touching nuclei can be defined and activated in the classifier.

In order to start a search on the Metafer MNScore system, up to 8 slides are loaded on the stage of the microscope. The correct operation mode is selected, and in a slide set-up dialogue the slide names are entered, the classifier is selected, and the search window is defined.

After starting the search, the system automatically engages a ×10 objective lens, adjusts the microscope lens according to the actual contrast and performs an autofocus. Subsequently, the complete search window is scanned, and positive objects are displayed in an image gallery. Simultaneously, a histogram is created, displaying the number of cells with 0, 1, 2 . . . MN (Figure 2A–C).

The gallery and the histogram can be used to perform a visual inspection of the scan results. It is, for example, easily possible to selectively display the positive BNC in the gallery and to correct false-positive MN counts.

The current stage of application. The Metafer MNScore system for automated image analysis scoring of MN is used in routine diagnostics [e.g. in preclinical phases of drug development (16)] and in research. Results obtained in three major fields of investigation can be found in recent publications.

Cancer research (2004). Testing of the system and comparison of results with visual analysis was performed on peripheral lymphocytes of breast cancer patients and controls (12,17). In these case–control studies, radiosensitivity was assessed after irradiation of lymphocytes by 2 Gy of γ-rays (challenge) in 91 breast cancer cases and 96 female controls. A subgroup (32 patients and 21 controls) was reanalysed by automated image analysis where the mean frequency of MN induced by 2 Gy of γ-rays was 150.3 ± 6.0 versus 102.4 ± 6.9 in patients versus controls, respectively. In general, significant differences between breast cancer cases and female controls after adjustment for age were found. Comparison showed higher MN frequency levels in visual counting than in automated image analysis, but both counts were highly positively correlated. Another case–control study focused on patients with sporadic prostate cancer (18). Comparison of baseline and induced MN frequencies (without and with irradiation) in 22 patients and 43 controls indicated that increased radiosensitivity is not a property of prostate cancer patients in general in contrast with above-mentioned breast cancer patients and controls. Two classifiers by Varga *et al.* (18) were applied in the prostate study with similar results.

Biomonitoring studies in the field of air pollution (2009). A new field for application of automated image analysis was opened in 2009, when the results from a biomonitoring study, assessing the effect of exposure to carcinogenic polycyclic aromatic hydrocarbons (c-PAHs), represented mainly by benzo[*a*]pyrene (B[*a*]P), in ambient air on the frequency of

Fig. 1. The automated scanning system Metafer with 80-position slide feeder.

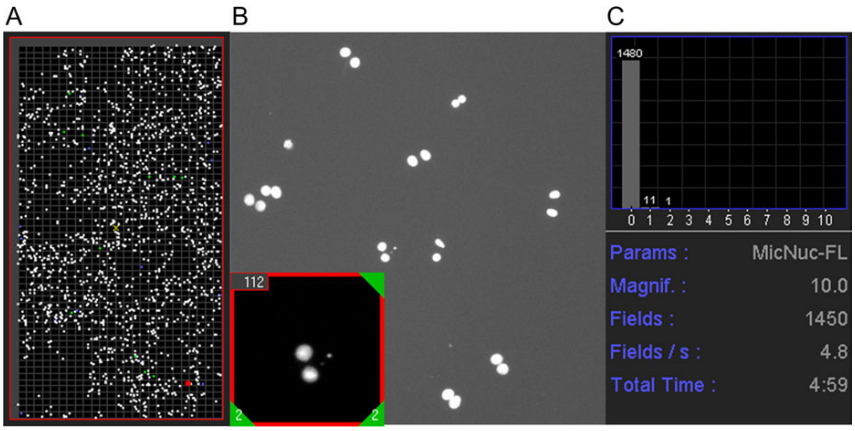

Fig. 2. Images of results of automated image analysis of MN: (**A**) distribution of BNC objects on a scanned slide, (**B**) live image of distribution of cells and inset #112 shows one BNC with two MN, taken from a gallery of images and (**C**) histogram with results and information on scanning.

MN in a group of city policemen from Prague sampled in February and May 2007 were published (19). In the same year, data from another study on bus drives and administrative workers monitored in Prague in November 2006 were obtained (20).

In Table I, we summarised the data mentioned above (Studies 1 and 2) and the new results unpublished so far (Studies 3–5) obtained until the beginning of 2010 in our laboratory, to show the main results and to demonstrate the ability and advantages of the automated system in biomonitoring studies. The data presented in the table were calculated from the automated scores corrected for false positives. The table shows the pooled data from 529 subjects obtained by the automated image analysis in studies carried out in years 2006–10. In these studies, the exposure to c-PAHs was measured by personal and/or stationary monitoring. Personal monitoring assessed the individual exposure to air pollutants 48 h before the collection of biological samples. Stationary monitoring by versatile air pollution samplers supplemented data from personal monitoring. In all studies, the DAPI staining of slides and a classifier setting very similar to that in the study by Varga *et al*. (12) was used. Here, we present the main information from above-mentioned projects.

Studies 1 and 2. The impact of air pollution in different seasons on the frequency of MN in binucleated lymphocytes is shown. In the first published study (19), Study 2, the group of 56 city policemen (20 smokers and 36 non-smokers) from Prague sampled in February and May 2007 were followed. The concentration of B[*a*]P by personal monitoring was 1.04 ± 0.76 ng/m^3 and 0.24 ± 0.54 ng/m^3 in February and May, respectively. The statistically significant decrease of concentrations of c-PAHs, B[*a*]P and frequencies of MN ($P < 0.001$) was observed when both months were compared. The study confirms that there are no differences in frequencies of MN between smokers and non-smokers and demonstrates the ability of c-PAHs to increase MN frequency, even if the exposure to c-PAHs occurred up to 60 days before the collection of biological material.

These data in Study 2 were replenished by previously sampled groups, focused on genetic damage in a group of 50 bus drivers and 50 administrative workers followed in November 2006 (20). The level of B[*a*]P measured by personal monitoring in a group of bus drivers was 1.04 ± 0.76 ng/m^3 (the same as in policemen sampled in February 2007). The slightly higher frequency of MN corresponded with higher age in a group of bus drivers when compared with policemen. The administrative workers, who spent the time prevalently indoor, had lower frequency of MN due to lower exposure to B[*a*]P assessed by personal monitoring (0.75 ± 0.36 ng/m^3). Besides these results, the comparison with conventional cytogenetic analysis (CCA) was presented. The data indicate the high sensitivity of MN analysis by automated system probably due to higher number of evaluated objects than in CCA. The automated image analysis showed the ability to reveal the differences in frequency of MN induced by personal exposure B[*a*]P between 0.24–1.04 ng/m^3.

Study 3. The data obtained in the project 3 involved asthmatic and healthy children ($N = 175$) aged 6–15 years. The study was performed in November 2008 in the Ostrava region. Personal monitoring was not carried out in this study but concentrations of air pollutants were very high. The level of B[*a*]P measured in November 2008 by stationary monitoring of the Health Institute in Ostrava-Bartovice was 11.38 ± 9.84 ng/m^3 (http://www.chmi.cz/uoco/isko/tab_roc/2008_enh/cze/pollution_overview/overview_BaP_TOBAP_512225.html). In this study, we did not find the statistically significant differences in the frequency of MN per 1000 BNC between asthmatic and healthy children. The lower levels of MN are in correspondence with younger age of these groups. The comparison between genders shows slightly higher, but not statistically significant difference in the level of MN in girls ($N = 83$, 3.33 ± 1.36 MN per 1000 BNC) versus boys ($N = 92$, 3.16 ± 1.29 MN per 1000 BNC). This trend is in agreement with previously published data on the effect of gender on MN frequency (21). The separate analysis in asthmatic and control children showed a significant difference in the level of MN between both genders in the control group only [girls ($N = 50$, 3.39 ± 1.17 MN per 1000 BNC) versus boys ($N = 44$, 2.91 ± 1.32 MN per 1000 BNC), $P < 0.05$].

Study 4. The group of city policemen from Prague was repeatedly investigated in February 2009 and 2010. Only

Table I. Overview of MN frequencies per 1000 BNC in the different sample groups assessed in the Department of Genetic Ecotoxicology by automated image analysis

Study	Sample group	Sampling period (month/year)	Place	*N*	Age (years) (mean ± SD)	BNC (mean in project)	MN/1000 BNC (mean ± SD)
1	Bus drivers	11/2006	Prague	50	50 ± 9	1000	8.48 ± 3.17[a]
	Administrative workers	11/2006	Prague	50	50 ± 11	1000	5.92 ± 2.82
2	Policemen	2/2007	Prague	56	34 ± 6	1000	7.32 ± 3.42[b]
	Policemen	5/2007	Prague	56	34 ± 6	1000	4.67 ± 2.92
3	Asthmatic children	11/2008	Ostrava	81	10 ± 3	3000	3.34 ± 1.41
	Healthy children	11/2008	Ostrava	94	11 ± 3	3000	3.16 ± 1.25
4	Policemen	2/2009	Prague	61	38 ± 8	3000	7.24 ± 2.27
	Policemen	2/2010	Prague	65	39 ± 8	3000	6.97 ± 2.11
5	Laboratory workers—A1	1(start)/2010	Prague	4	41 ± 13	6308	8.32 ± 1.63
	Laboratory workers—B1	1(start)/2010	Prague-Ostrava	4	51 ± 14	6118	7.96 ± 4.92[c]
	Laboratory workers—A2	1(end)/2010	Prague	4	41 ± 13	6485	8.47 ± 1.55
	Laboratory workers—B2	1(end)/2010	Ostrava-Prague	4	51 ± 14	4794	12.91 ± 6.49

N, number of subjects; A, spend all the time in Prague; B, spend 3 weeks in Ostrava (B1, before stay; B2, after stay).
[a]$P < 0.001$ (bus drivers versus administrative workers).
[b]$P < 0.001$ (policemen in February versus policemen in May).
[c]$P = 0.068$ (laboratory workers—B1 versus laboratory workers—B2).

non-smokers were involved in these groups. When no effect of smoking on the frequency of MN is taken into consideration, as confirmed in project 2, we can conclude that the results obtained for policemen sampled in February in three years (2007, 2009 and 2010) are relatively consistent. Personal exposure to B[*a*]P in these years was 1.04 ± 0.76 ng/m^3 (year 2007) 0.80 ± 0.55 ng/m^3 (year 2009) and 2.04 ± 1.87 ng/m^3(year 2010). The data of personal exposure to B[*a*]P in February 2010 are substantially higher than those measured in previous years, but the levels of MN do not differ from previous measurements. These results support the idea of the important role of exposure to c-PAHs in a certain period before the collection of samples. We suggest to analyse the impact of c-PAHs and B[*a*]P measured by stationary monitoring up to 60 days before the collection of samples as well as the influence of other factors (e.g. life style).

Study 5. The data presented in the study 5 further support the idea of a substantial impact of c-PAHs on the frequencies of MN. Even though in this study, only eight subjects participated (four spent all the time in Prague and four in the Ostrava region), a clear increase in MN frequency was observed in all subjects of Group B, who spent 3 weeks in the Ostrava region (probably the most polluted area in the European Union, January 8–28, 2010). The personal monitoring of exposure to environmental pollutants was not performed for these subjects, but the dramatic situation in the Ostrava region can be illustrated on the personal exposure data from other groups investigated in this region at the same time. The average concentrations of c-PAHs and B[*a*]P were 99.84 ± 92.96 ng/m^3 and 14.71 ± 13.31 ng/m^3, respectively.

Besides other results, the data mentioned above illustrate the impact of age on the frequency of MN. The impact of age is summarised in detail in Figure 3, where the data from exposed non-smoker 445 subject (characteristics in Table II) of both genders sampled in seasons characterised by higher levels of air pollutants (fall and winter) were pooled ($R = 0.59$, $P < 0.001$). The data obtained in our laboratory seem to be very similar to the baseline frequency of MN obtained by visual scoring in The International Collaborative Project on Micronucleus Frequency in Human Populations [Human MicroNucleus project (HUMN project)] (21) (http://www.humn.org). The additional checking of all galleries of randomly chosen slides revealed 10% false-negative BNC with MN. Regarding false-positive objects, all findings were checked and corrected. Thus, our results are not encumbered by this mistake.

These data show that automated Metafer MNScore is capable of evaluating high numbers of BNC per subject. While in early projects, the 1000 BNC per subject was evaluated, later the number increased to 3000 BNC per subject. The evaluation of higher numbers of BNC allows improvement of statistical power, which is important in all projects and gives a possibility to evaluate smaller groups as demonstrated in Study 5.

Radiation biodosimetry (2009 and 2010). At the turn of years 2009 and 2010, a couple of publications on radiation biodosimetry were published (22,23). In these studies, authors proposed the Metafer MNScore system for scoring of MN for population triage in a case of large-scale radiation events. This proposal is a result of an experiment, in which the levels of MN by automated image analysis were measured in blood samples from five males and five females after irradiation with different doses of γ-rays up to 3 Gy. The study resulted in similar MN rates per 1000 cells as found after irradiation of human lymphocytes with various doses of X-rays by Schunck *et al.* (11). The dose–response curve fitted to the average, automated MN scores of the 10 donors, showed that a dose of 1 Gy can be detected with an accuracy of 0.2 Gy (23). Even though the authors state that the dicentric assay provides a higher sensitivity in the first triage circumstances, the precision of the MN test is suggested as a better option when the fact of productivity is taken into consideration. They estimate that two technicians can process at least 60 samples in a 12-h shift. The study also provides results of a multicenter setting in two laboratories with additional donor samples. This simulation of a small radiation accident demonstrated that the estimated doses were very close to the real doses. The dose estimations

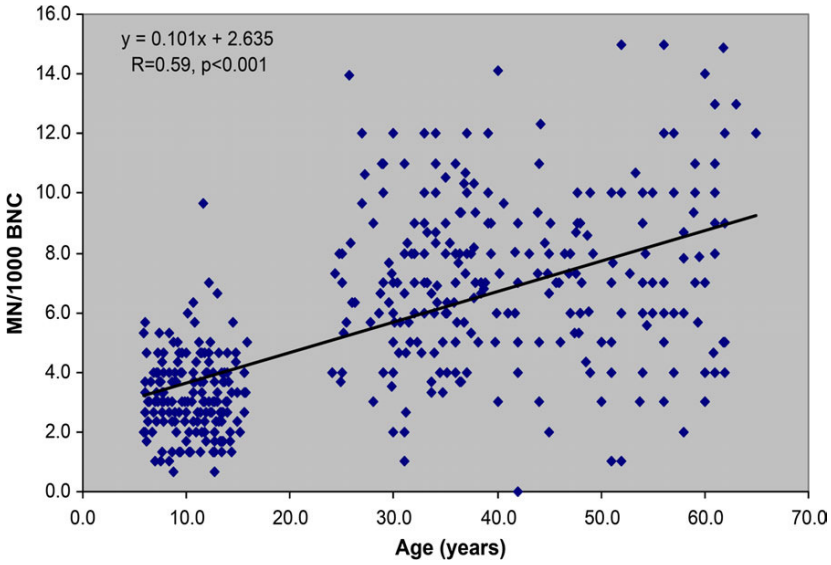

Fig. 3. The impact of age on the frequency of MN per 1000 BNC in 445 exposed subjects measured by automated image analysis using Metafer MNScore.

were in most cases within the real dose ± 20% (±0.4 Gy). The inter-laboratory differences observed are only minor and are probably due to slight discrepancies in cell culture conditions, cell fixation or slide preparation techniques. As most other users did, and also the manufacturer recommends, authors opted to use DAPI staining for automated scoring since Giemsa stained slides generate too many artifacts.

Validation of the system by visually checking the BNC gallery in this study, using published classifier settings, revealed 6.28% of false BNC mainly consisting of apoptotic cells or two mononuclear cells lying close together. The verification of correctly identified MN showed only 1% false positives and 1% of false negatives. The comparison between visual count of MN and automated image analysis showed a very strong correlation by linear regression analysis ($r^2 = 0.917$) as well as by χ^2 goodness of fit test (χ^2 of 7.770 with $P = 0.169$).

Restrictions of automation

In all published studies, the automated image analysis is obviously compared with visual analysis, which started in 1985, when cytochalasin-B was first used to inhibit cytokinesis (24). During the years, analysis of MN was improved (25), described in detail in (26) and verified and applied in innumerable studies. In comparison with long history of the visual analysis and many successful studies (e.g. the HUMN project) (http://www.humn.org) (26,27), results on automated image analysis are relatively new, but they clearly show that automation has advantages in large-scale studies.

Some important restrictions, however, were mentioned recently by Fenech (28) and were also reported by users of automated systems.

Table II. Characteristics of exposed non-smokers subjects in winter season

$N = 445$	Age (years)	MN/1000 BNC
Mean	29.50	5.63
Median	31.02	5
Std. Deviation	17.53	3.01
Minimum	6	0
Maximum	65	15
Percentiles		
25%	11.85	3.33
50%	31.02	5
75%	43.98	7.67

1. In all studies, the automatic image analysis was used to evaluate genetic damage by the frequency of MN in BNC. In some studies that used visual analysis, various structures and cells with different viability status were scored. The automated system does not evaluate events such as nucleoplasmic bridges, nuclear buds, necrotic and apoptotic cells.
2. The ability of the system to detect BNC with and without MN is slightly limited by the fact that it is difficult to detect MN, which are in contact with the nuclei. This is, as some authors discuss, probably one of the explanations why the levels of MN by automated analysis are generally lower than the manually obtained data.
3. The draft of the OECD guideline on scoring cytokinesis-block MN assays (29) includes the calculation of the cytokinesis-block proliferation index. Since the Metafer MNScore system is focused on the analysis of BNC or mononucleated cells, this calculation cannot be done automatically. There are, however, solutions to implement an automated analysis of the number of nuclei within each cell, if a fluorescent staining of cytoplasm and nuclei is applied (P. Haub and C. Schunck, unpublished results).

Recommendations for future research

1. Data published by users of the automated MN image analysis clearly show that there are large differences in lymphocyte culture conditions and processing of samples (12,19,23). It is likely that these differences also have impact on the optimisation of classifier settings (Table III) and on the results. Thorough description of the method including every single detail of the protocol should therefore accompany the results from each laboratory, so that it can be easily replicated in other laboratories. We are together with other authors convinced that this strategy, accompanied with the open exchange of classifiers between labs, is a crucial step toward a standardisation of automated MN scoring (23).
2. Different studies show clearly that the application of fluorescent staining by DAPI for evaluating genetic damage by visual counting of MN is less common because of the invisible cytoplasm. The application of this staining, however, has obvious advantages if automated image analysis is used (15–18). The combination of DAPI staining with different fluorescent probes (centromeric, telomeric or locus specific) may provide the chance to obtain the additional information about the mechanism of MN formation.

Table III. The comparison of classifier settings in studies that used the automated image analysis of MN in human lymphocytes

Object	Parameter of classifier	Study 1 (Varga *et al.* 12,18)	Study 2 (Rossnerova *et al.* 19,20)	Study 3 (Willems *et al.* 23)
Nuclei	Object threshold (in %)	20	20	15
	Minimum area (μm^2)	40	40	80
	Maximum area (μm^2)	2000	2000	1000
	Maximum relative concavity depth	0.12	0.12	0.16
	Maximum aspect ratio	1.50	1.50	1.37
	Maximum distance (μm)	18	18	25
	Maximum area asymmetry (in %)	A—75, B—90	90	80
	Region of interest radius	40	30	30
	Maximum object area in region of interest (μm^2)	20	20	35
MN	Object threshold (in %)	5	15	7
	Minimum area (μm^2)	1	1	1
	Maximum area (μm^2)	21	21	40
	Maximum relative concavity depth	1.0	1.0	0.7
	Maximum aspect ratio	1.72	1.72	1.70
	Maximum distance	29	29	25

'A' and 'B' indicate two different settings (percentages) of maximum area asymmetry that were tested in studies 12 and 18.

3. All mentioned studies concern the evaluation of MN in human peripheral lymphocytes, but there is also huge field for application in different cell lines in *in vitro* studies, and there are already laboratories using the system for CHO and mouse cells (16). The possibility to modify classifier settings for these cell lines is an advantage. With the software MetaCyte, installed on the same Metafer platform, it is also possible to automatically measure MN in other tissue *in vivo* such as erythrocytes and buccal cells. One of the main advantages of the Metafer MNScore system is the possibility to evaluate high numbers of BNC. The scanning of one slide lasts ~3 to 5 min irrespective of the density of objects. Thus, this platform gives a possibility to increase the statistical power of results, when the maximum of BNC is evaluated, though still a lot of time is saved when compared with visual analyses (23).

4. Once standardised preparation protocols and classifiers are available, it will be possible to collect, compare and summarise data from different laboratories. The collection of such results could be a task for a future international project focused on assessment of MN by automated image analysis, which could form the basis of a new HUMN project focused on automated measurement of MN in human lymphocytes and a new $HUMN_{xL}$ project focused on automated MN measurement in human exfoliated buccal cells (http://www.humn.org). At the moment, an FP7 project Multibiodose (Multidisciplinary biodosimetric tools to manage high-scale radiological casualties) is ongoing which has as a main goal to establish a biodosimetric network that will be able to respond in case of a mass casualty situation.

Concluding remarks

Despite the restrictions mentioned above, it can be concluded that automated image analysis of MN using the Metafer MNScore system is a reliable tool for the assessment of chromosomal damage. It allows for analysis of large numbers of cells, with the additional advantage of limited subjectivity and lack of scoring bias, which are so critical for visual scoring.

The Metafer MNScore system facilitates high-throughput MN scorer and offers the option of storing images for documentation. It, therefore, is a promising tool for improving risk assessment of human populations exposed to environmental or occupational mutagens, which is crucial for prevention of cancer and for the high-throughput biodosimetry following to large-scale radiation accidents.

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Příloha 8:

Rossnerova, M. Spatova, P. Rossner Jr., Z. Novakova, I. Solansky, R.J. Sram

Factors affecting the frequency of micronuclei in asthmatic and healthy children from Ostrava

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Factors affecting the frequency of micronuclei in asthmatic and healthy children from Ostrava

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ABSTRACT

A higher incidence of asthma is one of the serious problems confronting urban populations worldwide. The aim of the present study was to analyze the effect of age, gender, smoking, vitamin intake, genetic polymorphisms in genes related to the metabolic activation of polycyclic aromatic hydrocarbons (PAHs) and their detoxification and oxidative damage to DNA, lipids and proteins on the frequency of micronuclei (MN) in a group of 175 children (81 with bronchial asthma and 94 healthy controls) aged 6–15 years. The study group from the most polluted region of the Czech Republic, Ostrava, was followed in November 2008, when the mean concentration of benzo[a]pyrene (B[a]P) measured by stationary monitoring was 11.4 ± 9.8 ng/m^3. The results of cotinine analysis revealed active smoking in 15 children. The frequency of MN per 1000 binucleated cells (MN/1000 BNC), measured by automated image analysis, indicated a significant risk for smoking children with asthma in comparison with smoking control children (4.25 ± 1.54 and 3.00 ± 0.77, respectively, $p < 0.05$). Girls in the control group had 16% higher levels of MN in comparison with boys. Markers of oxidative damage to DNA, proteins and lipids were not associated with asthma in this study. Higher levels of MN were associated with increased levels of protein carbonyl groups. We conclude that smoking asthmatic children are at higher risk of DNA damage measured as the frequency of micronuclei in peripheral blood lymphocytes.

1. Introduction

Asthma in children is a commonly studied topic as evident from the number of references, more than 34,000, found using the keywords "asthma" and "children" in the PubMed database as of the beginning of 2011. The incidence of asthma as well as the risk of allergic diseases and increased morbidity are higher in urban areas than in rural locations. The hypotheses for this uneven distribution include the lack of exposure in city areas to natural factors; such as farm animals; pets and non-pasteurized milk; that can positively promote the development of the immune system [1]. City inhabitants are affected by various factors including environmental pollution; stress and diet. Besides a genetic predisposition; the development of asthma is affected by factors that differ by location: air pollution; socioeconomic aspects; environmental tobacco smoke exposure and exposure to cockroach; mouse; and rat allergens [2,3].

It is generally accepted that children are more sensitive to air pollution, mainly due to the fact that their immune system and lungs are not fully developed yet [4]. The ubiquitous carcinogenic polycyclic aromatic hydrocarbons (c-PAHs) bound to respirable air particles of particulate matter are among the most studied environmental pollutants with deleterious effects on human health [5]. They are present mainly in industrial, heating and traffic emissions. The International Study of Asthma and Allergies in Childhood (ISAAC) evaluated the associations between truck traffic pollution on residential streets and symptoms of asthma and allergic diseases in developed and developing countries. The results, based on self-reported truck traffic, indicate a global association between asthma and greater exposure to air pollutants [6].

The effect of air pollution on human health has been evaluated in many studies by a set of biomarkers, which have been tested and updated over the years [7,8]. The three main categories of biomarkers include biomarkers of exposure, susceptibility

Abbreviations: 8-oxodG, 8-oxo-7,8-dihydro-2′-deoxyguanosine; 15-F_{2t}-IsoP, 15-F_{2t}-isoprostane; B[a]P, benzo[a]pyrene; BNC, binucleated cell; c-PAHs, carcinogenic polycyclic aromatic hydrocarbons; DAPI, 4′-6-diamidino-2-phenylindole; ELISA, enzyme-linked immunosorbent assay; EPHX1, epoxide hydrolase; GSTM1, GSTT1, glutathione-S-transferase M1, T1; HPLC, high performance liquid chromatography; ISAAC, International Study of Asthma and Allergies in Childhood; MN, micronuclei; PCR, polymerase chain reaction; ROS, reactive oxygen species.

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and effect. Exposure data are obtained by personal or stationary monitoring, while individual susceptibility data are obtained by analyzing genetic polymorphisms, particularly in genes encoding enzymes related to the metabolic activation and detoxification of xenobiotics. Polymorphisms in the *EPHX1*, *GSTM1* and *GSTT1* genes were studied in various exposed and control groups in our previous studies [9–11]. Oxidative stress and cytogenetic markers are among the biomarkers of effect.

Oxidative damage is induced as a consequence of oxidative stress caused by reactive oxygen species (ROS) that attack biomolecules. Oxidative stress is caused by an imbalance between oxidants and antioxidants in the organism. ROS can attack nucleic acids and cause damage to bases, sugars and sugar–phosphate backbones. This leads to single-stranded or double-stranded breaks [12]. ROS also attack lipids and proteins. Lipids form reactive intermediates that propagate oxidative stress by reaction with other macromolecules within the cell [13]. Proteins are mostly oxidized on the side chains of the amino acids proline, arginine, lysine and threonine. The oxidation of proteins leads to the loss of their catalytic functions, their cross-linking and aberrant folding [14]. The oxidation of macromolecules is connected with the development of cancer, lung and cardiovascular diseases [15]. The levels of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG), the most frequently analyzed marker of oxidative damage to DNA, were recently studied in the Czech Republic in children from the industrial region of Teplice and the agricultural region of Prachatice. Slightly higher levels of 8-oxodG were detected in asthmatic children from Teplice [16].

The investigation of chromosomal damage in human peripheral lymphocytes by micronuclei assay is a common method used in numerous studies, including in vivo studies focused on the impact of various occupational exposures, smoking, exposure to pesticides, air pollution or various drugs. The special issue of Mutagenesis devoted to micronuclei assays, with a set of mini-reviews summarizing various scientific efforts in this field, is the best example of the usefulness of the method. Moreover, the issue provides an update on current and future methods, e.g. automated image analysis [17]. Studies focused on the relationship between bronchial asthma and the levels of micronuclei are scarce. The study by Herrström et al. [18] analyzed the frequency of MN in the B-, T4- and T8-lymphocytes of 12 boys and 5 girls with asthma. They found a significantly increased frequency of MN in B-lymphocytes in asthmatic children. The level of cytogenetic damage in healthy children from the Teplice and Prachatice regions, with different levels of air pollution, was examined in a pilot study [19]. Pedersen et al. [19] detected higher levels of visually analyzed micronuclei in children from Teplice in comparison with groups from the rural district of Prachatice.

Bronchial asthma is the most common inflammatory disease of the respiratory system which impacts the immune response of the organism. Oxidative stress plays a critical role in the pathogenesis of asthma. Higher levels of the molecules involved in enhanced oxidative stress were found in biological samples taken from asthmatic patients compared with normal control subjects. Higher incidences of bronchial asthma have been reported in areas with air pollution, which is a representative stimulus among exogenous oxidants [20].

In our study, we hypothesize that the immune reactions increase oxidative damage to macromolecules, which in turn impacts genetic damage as represented by the frequency of micronuclei. We evaluated the level of DNA damage in groups of asthmatic and healthy children from Ostrava Radvanice–Bartovice, the area with the highest levels of c-PAHs in the Czech Republic and an increased incidence of bronchial asthma as documented in health records from the years 2001–2007. The current average countrywide asthma incidence is 8–10%, but in the Ostrava Radvanice–Bartovice region it is increased to 30%. For comparison, in the Teplice and Prachatice regions studied previously, the incidence of bronchial asthma in 2006 was 8.8% in the industrial region of Teplice and 5.5% in the rural region of Prachatice [21]. The level of genetic damage was measured by the automated image analysis of MN in a group of 175 children. In addition, we analyzed oxidative damage to DNA, proteins and lipids. Finally, the impact of individual susceptibility to the negative effects of air pollutants, lifestyle and other factors was included in the analysis.

2. Materials and methods

2.1. Study subjects

The study was performed in Ostrava Radvanice–Bartovice in November 2008. The study population was a group of 81 children with bronchial asthma and 94 control children. The average age of the participants with asthma and the healthy controls was 10.4 ± 2.8 and 10.8 ± 2.6 years, respectively. The total group of 175 children included 92 boys and 83 girls. According to the questionnaires, 78 children were from nonsmoking households and 97 from smoking households where up to five persons smoked.

Questionnaires on personal medical history and life-style were obtained from all participants. The parents of the children signed an informed consent form and could cancel their participation at any time during the study, according to the Helsinki II declaration. The ethical committee of the Institute of Experimental Medicine AS CR in Prague approved the study.

Blood, urine and saliva samples were collected for laboratory analysis. Blood was drawn by venipuncture into vacuettes containing sodium heparin (for the micronucleus test), lithium heparin (for the vitamin C assay) or EDTA (for the vitamin A, E and oxidative damage of proteins and lipids assays). Urine was used to check tobacco smoke exposure and to measure the level of 8-oxodG, while saliva was used for DNA isolation and genotyping. The samples were placed at 4 °C and immediately transported by a messenger service to the Department of Genetic Ecotoxicology in Prague for further processing.

2.2. Monitoring of air pollution

The concentrations of B[a]P in Ostrava-Bartovice in 2008 were measured by the Health Institute in Ostrava using stationary monitoring, and the data were obtained from the Czech Hydrometeorological Institute [22].

2.3. Micronucleus test

Whole venous blood cultures were established within 24 h after blood collection. The description of the processing of samples based on the cytochalasin B blocking of cytokinesis [23] was published previously [24].

The microscopic analysis of DAPI-stained slides was done by automated image analysis [25]. The automated scanning system Metafer 4, Version 3.2.1, from MetaSystems (Altlusshein, Germany) with a motorized Axio Imager Z1 microscope (Carl Zeiss, Germany) was used [26]. Scanning of binucleated cells (BNC) was performed at a final magnification of 100×. The classifier settings (MicroNuc Classifier, Version 3.5.104) for nuclei and micronuclei image processing operations were the same as in our previous studies [17]; 3000 BNC per subject were analyzed. All automated findings with one or more MN were checked and corrected if necessary by one person. The results were calculated as mean MN per 1000 BNC (MN/1000 BNC).

2.4. Cotinine and vitamin assays

Urinary cotinine levels were analyzed by radioimmunoassay [27]. Children with cotinine levels greater than 450 ng/mg of creatinine were considered active smokers.

Vitamin A, C and E levels in plasma were analyzed in a service laboratory of the Institute of Clinical Biochemistry and Laboratory Diagnostics of the General University Hospital in Prague by HPLC [28,29].

2.5. GSTM1, GSTT1 and EPHX1 genotyping

DNA samples from saliva were isolated by the ORAgene DNA kit (DNA Genotek, Ontario, Canada) according to the manufacturer's protocol.

The presence or absence of the *GSTM1* and *GSTT1* genes was assessed by the TaqMan Real-Time PCR Assay (Applied Biosystems, Carlsbad, CA, USA; TaqMan Gene Copy Number Assays [PN4331182]). The method allows determining the exact number of genes in the sample, but for the purposes of our study we split the samples into two groups: *GSTM1/GSTT1* null and *GSTM1/GSTT1* positive samples (all samples having at least one copy of the gene). The assay was performed on a 7900HT system (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's recommendation.

EPHX1 polymorphisms in exon 3 (*EPHX1-3*) and in exon 4 (*EPHX1-4*) were also analyzed by the TaqMan Real-Time PCR Assay (TaqMan Drug Metabolism Genotyping Assays [C_14938_30 for exon 3 and C_11638783_30 for exon 4]). A detailed

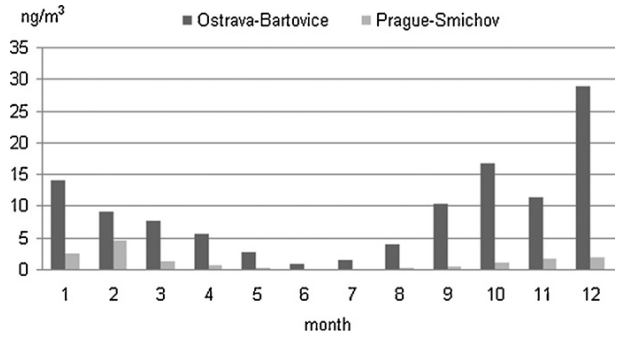

Fig. 1. Mean monthly concentrations of B[a]P measured by stationary monitoring in Ostrava-Bartovice (high levels of air pollution) and Prague–Smichov (medium levels of air pollution) in 2008.

description of different EPHX1 activities defined as the various combinations of *EPHX1-3* and *EPHX1-4* genotypes was published previously [9].

2.6. Oxidative damage assay

Levels of 8-oxodG in urine were analyzed by a competitive ELISA as previously described [30,31]. Each sample was analyzed in triplicate. The urinary 8-oxodG concentration was expressed as nmol 8-oxodG/mmol creatinine.

Levels of protein carbonyl groups were assessed in blood plasma using a non-competitive ELISA [30], with some modifications [32]. Each sample was analyzed in triplicate. The plasma protein carbonyl concentration was expressed as nmol carbonyl/ml plasma.

Plasma 15-F_{2t}-isoprostane (15-F_{2t}-IsoP) levels were analyzed using an immunoassay kit from Cayman Chemical Company (Ann Arbor, MI, USA) according to the manufacturer's protocol. The details were described previously [33]. Each sample was analyzed in duplicate. The 15-F_{2t}-IsoP concentrations were expressed as pg 15-F_{2t}-IsoP/ml plasma.

2.7. Statistical analysis

Statistical analyses were carried out using SAS 9.1.3. software (SAS Institute, NC, USA). For the data that were not distributed normally, the nonparametric Mann–Whitney Sum *U*-test was used for the comparison between groups. Bivariate logistic regression was performed to assess the relationship between MN frequencies and markers of oxidative damage. Multivariate logistic regression assessed the impact of the measured biomarkers in asthmatic children in comparison with the control group.

3. Results

The study was performed in the Ostrava region in the Czech Republic where the concentrations of B[a]P during the year are mostly elevated. Fig. 1 shows a comparison of the mean concentrations of B[a]P in individual months measured by stationary monitoring in highly (Ostrava-Bartovice) and moderately (Prague–Smichov) polluted locations. The level of 1 ng/m^3 of B[a]P in the ambient air – the planned yearly emission limit for the year 2012– was met in Ostrava-Bartovice only in June; from 61 measurements conducted at a frequency of one measurement per 6 days, this limit was exceeded 51×. The maximum concentration of B[a]P was 92 ng/m^3.

Table 2
MN frequencies (mean ± SD) per 1000 BNC in the studied groups by the level of cotinine.

Group	*N*	Cotinine/creatinine (ng/mg)	MN/1000 BNC
Nonsmokers			
All	160	28.2 ± 41.0	3.20 ± 1.33
Asthmatics	73	20.4 ± 41.3	3.23 ± 1.38
Controls	87	28.8 ± 40.9	3.18 ± 1.29
Smokers			
All	15	1622.8 ± 866.4	3.67 ± 1.36
Asthmatics	8	1581.6 ± 695.1	4.25 ± 1.54*
Controls	7	1669.9 ± 1087.6	3.00 ± 0.77

N: number of subjects.
* $p < 0.05$ (asthmatic smokers vs. control smokers).

In Table 1 the basic characteristics of the asthmatic and healthy children (number of subjects, mean, standard deviation (SD), median, maximum and minimum) for age, cotinine levels and vitamin A, C and E levels are presented. The data show similar ages and cotinine levels in both groups but differences in the level of vitamin C (lower intake in asthmatics, $p < 0.01$). The maximal levels of cotinine indicate the presence of actively smoking children in both groups.

The frequencies of MN were calculated in all 175 children and compared: asthmatic vs. controls, boys vs. girls, children up to 10 years vs. children above 10 years, asthmatic children and controls divided by exposure to familiar smoking. The differences between the compared groups were not significant (data not shown); however, they indicated that there is a slightly higher frequency of MN in asthmatic children in comparison with controls, slightly greater genetic damage in girls in comparison with boys (this difference is significant in the control subgroup: boys 2.91 ± 1.32 MN/1000 BNC vs. girls 3.39 ± 1.17 MN/1000 BNC, $p < 0.05$), and a similar frequency of MN in young children (8.0 ± 1.2 years) and in older children (12.7 ± 1.5 years). The comparison of children based on exposure to familial smoking showed greater, but not significant, DNA damage in asthmatic children from smoking families (3.40 ± 1.34 MN/1000 BNC) in comparison with control children from nonsmoking families (3.06 ± 1.15 MN/1000 BNC).

Some of the children in both subgroups had substantially elevated levels of cotinine, which corresponded to the levels measured in active smokers. The frequencies of MN separated by the cotinine levels are presented in Table 2. The new subgroup of active smokers consisted of 15 children with cotinine levels above 450 ng/mg creatinine. The frequencies of MN in 8 asthmatic children classified as active smokers was significantly higher in comparison with control smoking children ($p < 0.05$). The results in Tables 3 and 4 are shown after smoking children were excluded.

Table 3 summarizes the frequencies of MN in subgroups distinguished by their genotypes in the *GSTM1*, *GSTT1* and *EPHX1* genes, as assessed in 156 children. A significantly higher frequency of MN was found in children carrying at least one allele of the *GSTM1* and *GSTT1* genes. No significant differences were found between different activities of the EPHX1.

Table 1
Characteristics of the study subjects.

	Asthmatics			Controls			*p*
	N	Mean ± SD	Median (range)	*N*	Mean ± SD	Median (range)	
Age (years)	81	10.4 ± 2.8	10.4 (5.9–15.9)	94	10.8 ± 2.6	11.0 (6.0–15.6)	0.32
Cotinine/creatinine (ng/mg)	81	180.9 ± 511.4	11.55 (1.9–2781.3)	94	151.0 ± 515.2	13.6 (2.9–3345.1)	0.79
Vitamin A (mg/l)	81	0.7 ± 0.3	0.7 (0.2–1.5)	93	0.7 ± 0.3	0.60 (0.2–1.9)	0.07
Vitamin C (mg/l)	63	6.2 ± 2.6	5.7 (1.9–13.4)	46	7.5 ± 2.8	7.2 (1.3–13.4)	<0.01
Vitamin E (mg/l)	81	11.0 ± 4.2	10.6 (3.1–23.0)	93	9.8 ± 3.0	9.9 (3.0–19.9)	0.09

N: number of subjects.

Table 3
MN frequencies (mean ± SD) per 1000 BNC in nonsmoking children by genotype.

Studied gene	Genotype/Activity	*N* (%)	MN/1000 BNC	*p*
GSTM1	Positive	87 (55.7%)	3.46 ± 1.45	0.02
	Negative	69 (44.3%)	2.90 ± 1.11	
GSTT1	Positive	117 (75%)	3.36 ± 1.42	0.02
	Negative	39 (25%)	2.79 ± 0.96	
EPHX1	Low	62 (39.7%)	3.12 ± 1.12	0.87[a]
	Medium	80 (51.3%)	3.23 ± 1.48	0.39[b]
	High	14 (9%)	3.55 ± 1.41	0.34[c]

N: number of subjects.
[a] Low vs. medium activity of EPHX1.
[b] Medium vs. high activity of EPHX1.
[c] Low vs. high activity of EPHX1.

The levels of markers of oxidative damage to biomolecules are summarized in Table 4 (8-oxodG – a marker of oxidative DNA damage, carbonyls groups – a marker of oxidative damage of proteins and 15-F_{2t}-isoprostane – a marker of oxidative damage of lipids). Our results show similar levels of oxidative damage in both groups. The results from bivariate logistic regression of the frequency of MN and oxidative damage markers in all the children, with and without smokers, show a significant association between the level of MN and oxidative damage of proteins (Table 5). The results from multivariate logistic regression presented in Table 6 for the same groups indicate a slightly higher risk of an above-median frequency of MN in asthmatic children. The markers of oxidative damage in asthmatic children without smokers did not confirm an association with the diagnosis of bronchial asthma.

4. Discussion

In the present study we analyzed the levels of DNA damage in groups of asthmatic and healthy children from Ostrava Radvanice–Bartovice, locations where the concentrations of c-PAHs in ambient air substantially exceed the concentrations measured in others parts of the Czech Republic during the whole year. The effect of vitamin intake, exposure to tobacco smoke, parameters of oxidative damage and individual susceptibility to xenobiotics were also analyzed. Three thousand BNC per sample were analyzed by automated image analysis to obtain more statistically significant data, as suggested previously [24].

Our earlier results indicated the high sensitivity and reliability of the method when used for assessing the effect of low personal exposure to B[a]P [17,24]. A recent study from China [34] compared the results obtained in workers at a steel factory with those from a medical staff (the control group). The exposure to B[a]P was 926.9 ng/m^3 in exposed workers and 49.1 ng/m^3 in controls. Genetic damage was significantly higher in the exposed group than in controls. However, if we consider the relatively low genetic damage in the control group (3.98 MN/1000 BNC) and compare it with the reported exposure to B[a]P, which is extremely high when compared with our previously reported data [17], we can speculate about a non-linear relationship between the levels of pollutants and DNA damage in people living in polluted areas. The study by van Leeuwen et al. [35] that compare gene expression in children and adults living in different regions in the Czech Republic show significant differences in gene expression profiles. The preliminary results from a study comparing gene expression profiles in children from Ostrava and Prachatice lead to similar conclusions (unpublished data). These results could explain the non-linear relationship between the dose and the damage. Another very marked reaction was observed after the environment was changed due to moving to a place with high air pollution and the organism was exposed for a short time to high concentrations of air pollutants [17]. In agreement with this new knowledge, the level of genetic damage in our study group living in Ostrava, where the levels of B[a]P in Novem-

Table 4
Oxidative damage (mean ± SD) in nonsmoking children.

	N	8-oxodG (nmol/mmol creatinine)	*N*	Protein carbonyls (nmol/ml plasma)	*N*	15-F_{2t}-IsoP (pg/ml plasma)
All	160	6.38 ± 2.55	159	21.06 ± 4.29	159	161.07 ± 62.8
Asthmatics	73	5.96 ± 2.61	73	20.82 ± 3.55	73	160.04 ± 76.97
Controls	87	6.73 ± 2.46	86	21.27 ± 4.83	86	161.94 ± 48.08
p[a]		0.07		0.74		0.08

N: number of subjects.
[a] Asthmatics vs. controls.

Table 5
Bivariate logistic regression of the frequency of MN and markers of oxidative damage.

	All (with smokers)			All (without smokers)		
	N	OR[a] (95% CI)[b]	*p*	*N*	OR[a] (95% CI)[b]	*p*
8-oxodG	175	0.93 (0.51–1.70)	0.81	160	1.05 (0.56–1.98)	0.87
Protein carbonyls	174	2.40 (1.29–4.47)	0.006	159	2.47 (1.29–4.71)	0.006
15-F_{2t}-IsoP	174	0.62 (0.34–1.14)	0.12	159	0.56 (0.30–1.07)	0.08

N: number of subjects.
[a] Odds ratio.
[b] Confidential interval.

Table 6
Multivariate logistic regression of the measured biomarkers in asthmatic children in comparison with the control group.

	All (with smokers)		All (without smokers)	
	OR[a] (95% CI)[b]	*p*	OR[a] (95% CI)[b]	*p*
MN/1000 BNC	1.75 (0.94–3.29)	0.08	1.69 (0.87–3.30)	0.12
8-oxodG	0.54 (0.32–0.99)	0.05	0.64 (0.34–1.12)	0.17
Protein carbonyls	0.60 (0.33–1.07)	0.08	0.77 (0.43–1.48)	0.43
15-F_{2t}-IsoP	0.54 (0.29–0.98)	0.04	0.60 (0.30–1.17)	0.13

[a] Odds ratio.
[b] Confidential interval.

ber, as well as throughout the year, were substantially higher than in Prague (Fig. 1), is not as high as expected provided that a linear relationship between dose and effect exists as well as possible adaptive response.

It is generally accepted that children are more sensitive to air pollution than adults. Children are especially vulnerable to the effects of air pollution because their immune and respiratory systems are not yet fully developed [4]. The comparison of genetic damage between asthmatics and controls in our study indicates a similar exposure to pollutants in both groups.

Unexpectedly, in our study we found 15 children who were active smokers, eight of them diagnosed with bronchial asthma. A previous study [36] confirmed that MN frequency was not increased in moderate smokers; only heavy smokers (30 cigarettes or more per day) showed a significant increase in genetic damage as measured by the micronucleus assay. Our results obtained in control smoking children are in agreement with this study, but significantly higher levels of MN in smoking asthmatic children ($p < 0.05$) indicate the risk of a combination of both factors.

Our results obtained by automated image analysis indicate the influence of gender on MN frequency. Higher levels of DNA damage in girls are in agreement with published data for visual scoring [37,38]. MN frequency was 16% higher in girls in the control group, compared to control boys which is very similar to the results published by Bonassi et al. [37] in which females had a 19% higher MN frequency.

We did not find any significant differences comparing MN frequencies between children younger and older than 10 years. We can explain these results by the relatively narrow range of age. Pooled data from our laboratory [17] from 445 non-smoking subjects sampled in the winter season and aged from 6 to 65 demonstrate that the data obtained by automated image analysis do reflect the effect of age and are in agreement with the results of the HUman MicroNucleus project [37] as well as with those from other studies [38,39].

We observed significantly lower levels of vitamin C in asthmatic children than in controls. This is in agreement with the results of a recent meta-analysis, which reports that lower serum levels of vitamin C are associated with increased odds of asthma [40]. The ability of vitamin C to act as an antioxidant [41], thus decreasing oxidative damage to DNA and other macromolecules, may play a role.

The impact of individual susceptibility caused by genetic polymorphisms in selected metabolic genes on the frequency of MN was also studied. The frequencies of individual variants of the alleles corresponded with the expectations for a Caucasian population. The review by Iarmarcovai et al. [42] concluded that polymorphisms in *EPHX1*, *GSTT1*, and *GSTM1*, which were also studied in our work, are of special importance in modulating the frequency of chromosomal damage in individuals exposed to genotoxic agents and in unexposed populations. Nevertheless, the results from published studies are inconsistent and conflicting due to the low number of study subjects. A recent pooled analysis of data by Kirsch-Volders et al. [43] in 646 individuals indicates that the *GSTT1* negative subjects had lower MN frequencies than their positive counterparts in the total population, as in our study, and that the protective effect of this genotype is reversed with increasing age.

Oxidative damage could be one of the possible mechanisms responsible for the negative impact of bronchial asthma on genetic material associated with a higher frequency of MN. The origin and development of asthma is closely associated with inflammatory processes [44]. In some studies, higher levels of oxidative stress markers in asthmatic children were observed: increased concentrations of 8-isoprostane and malondialdehyde in exhaled breath condensate [45–47] and carbonylated proteins in allergic inflammation in humans [48]. However, in another study, sputum 8-isoprostane was not elevated in mild asthmatic children and adults [49]. The authors concluded that this biomarker may not be sensitive in reflecting oxidant burden in mild asthma. Another large study ($N = 589$) focused on evaluating the levels of 8-isoprostane and protein carbonyls in the plasma of young people [50]. Although the levels of oxidative stress-related biomarkers were detectable, the authors did not find any connection between oxidative damage and asthma, which is in agreement with our results. We can conclude that biological material such as plasma and urine are probably not optimal for analysis of biomarkers in these studies.

In conclusion, to the best of our knowledge this work is the first human biomonitoring study to find an elevated risk of genetic damage, expressed as MN levels, in smoking asthmatic children. The environment, with a high concentration of B[a]P in the ambient air, probably had no substantial impact on the level of MN, but additional study to compare genetic damage in asthmatic children in areas with low and high concentrations of B[a]P will be required to confirm this statement. Different biological materials, such as exhaled breath condensate or sputum, should be used in a future study analyzing oxidative stress in asthmatics.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Příloha 9:

Rossnerova, M. Spatova, A. Pastorkova, N. Tabashidze, M. Veleminsky Jr., I. Balascak, I. Solansky, R.J. Sram

Micronuclei levels in mothers and their newborns from regions with different types of air pollution

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Micronuclei levels in mothers and their newborns from regions with different types of air pollution

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ABSTRACT

The aim of this study was to analyze genetic damage in human lymphocytes measured using automated image analysis of micronuclei (MN) in a group of 178 mothers and their newborns from two locations in the Czech Republic. The concentrations of benzo[a]pyrene (B[a]P), particulate matter of aerodynamic diameter <2.5 μm (PM2.5) and benzene were measured by stationary monitoring in the winter season of 2008/2009 in the capital city of Prague and in Ceske Budejovice, a regional city in a rural area. The 3-month mean concentration of B[a]P before delivery was lower in Prague in comparison with Ceske Budejovice: 1.9 ± 0.5 ng/m^3 vs. 3.2 ± 0.2 ng/m^3 ($p < 0.001$). The opposite trend was found for PM2.5 and benzene: 27.0 ± 2.5 μg/m^3 and 2.5 ± 0.5 μg/m^3 vs. 24.5 ± 0.7 μg/m^3 and 2.1 ± 0.8 μg/m^3 ($p < 0.001$) for Prague vs. Ceske Budejovice, respectively. The average age of the mothers was 31 years (range, 18–49 years). The frequencies of MN per 1000 binucleated cells were 8.35 ± 3.06 vs. 6.47 ± 2.35 ($p < 0.001$) for mothers from Prague and Ceske Budejovice, respectively, and 2.17 ± 1.32 vs. 3.82 ± 2.43 ($p < 0.001$) for newborns from Prague and Ceske Budejovice, respectively. Other factors, including vitamin intake, exposure to tobacco smoke, body mass index (BMI) before pregnancy, the education level of the mothers and the impact of the mothers' and fathers' ages were analyzed in our study. The results suggest that the different sensitivity of the study groups to various mixtures of carcinogenic pollutants could be affected by significant differences in lifestyle factors. Possible higher genetic damage was analyzed in newborns of smoking mothers, and the birth weight of this group was 7.4% lower ($p < 0.05$) in comparison with the newborns of nonsmoking mothers. No impact of the age of the mothers or fathers on MN frequency in the newborns was observed.

1. Introduction

The extent of interest in the deleterious effects of different chemical compounds on pregnancy outcome as well as on different population groups from locations with different levels of air pollution is obvious when reading the recent literature. The harmful effect of various air pollutants on human health must be evaluated in a complex way, because other factors, such as diet, smoking or the age of the participants in the study, could impact the resulting genetic damage in the organism. The set of biomarkers that has been developed over the years for this purpose [1,2] include biomarkers of exposure, effect and susceptibility.

Among many pollutants in the ambient air, great interest is shown in those that are classified by the International Agency for Research on Cancer (IARC) as carcinogenic or probably carcinogenic to humans. Benzo[a]pyrene (B[a]P) belongs among the intensively studied pollutants classified by the IARC as a carcinogenic compound to humans (Group 1) [3]. B[a]P is one of the chemicals adsorbed to respirable particulate matter (PM). Recently published conclusions from European studies on long-term exposure to PM indicate a direct association with mortality, particularly from cardiovascular and respiratory diseases [4]. Volatile organic compounds (VOC) are also commonly present in the ambient air. Benzene, one of the VOC, is also classified by the IARC as carcinogenic in Group 1 [5], and according

Abbreviations: %AB.C., percentage of aberrant cells; B[a]P, benzo[a]pyrene; BMI, body mass index; BNC, binucleated cell; c-PAHs, carcinogenic polycyclic aromatic hydrocarbons; DAPI, 4′-6-diamidino-2-phenylindole; F_G/100, genomic frequency of translocations/100 cells; FISH, fluorescence in situ hybridization; HPLC, high performance liquid chromatography; IARC, International Agency for Research on Cancer; LBW, low birth weight, <2500 g; MN, micronuclei; PM2.5, particulate matter of aerodynamic diameter <2.5 μm; VAPS, versatile air pollution sampler; VOC, volatile organic compounds.

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to the WHO no safe level of exposure can be recommended [6].

The impact of exposure to environmental toxicants on children's health has been investigated in various human cytogenetic studies [7–11]. The impact of different levels of environmental contamination on micronuclei levels in newborns was documented in studies from Serbia by Milosevic-Djordjevic et al. [12,13]. The extensive environmental pollution caused by air strikes, resulting in the pollution of soil, water and air, caused dramatically high levels of MN in newborns born 12 months after the bombing. A drop in MN frequencies was observed in newborns born 18 months and 7 years after the bombing.

The frequency of MN can be significantly affected by dietary factors [14]. The recent study by Fenech et al. [15] reported that a low intake of calcium, folate, nicotic acid, vitamin E and β-carotene and a high intake of pantothenic acid, biotin and riboflavin are significantly associated with increased genome instability. The type of diet (vegetarian or non-vegetarian) does not seem to be crucial for the resulting frequency of MN [16,17]. The recent NewGeneris project focused on a study of the maternal diet during pregnancy and newborns health [18]. New results from this project in 98 pregnant women suggest that maternal intake of dark meat contributes to DNA damage in maternal and umbilical cord blood [19].

The impact of smoking, investigated by cotinine levels as the major metabolite of nicotine in venous blood, on the level of micronuclei in peripheral blood lymphocytes has been analyzed in many studies. Bonassi et al. [20] observed that MN frequency was not increased in moderate smokers; only heavy smokers (30 cigarettes or more per day) showed a significant increase in genetic damage as measured by the micronucleus assay. These results obtained by visual scoring were confirmed by automated image analysis in groups of policemen from Prague [21]. The results of a new study comparing the frequencies of MN in healthy children and children with bronchial asthma indicate a significant risk of DNA damage for smoking children with asthma in comparison with smoking healthy children [11]. An investigation of MN in cord blood obtained from mothers who smoked >10 cigarettes per day during pregnancy revealed that tobacco compounds are able to induce chromosomal losses and breaks in newborns [22].

The impact of age on the level of MN is well known [23]. This trend was recently also shown by automated image analysis [24]. The impact of the age of parents on genetic damage in newborns was repeatedly analyzed in cytogenetic studies. A significant increase in the levels of stable chromosomal aberrations represented by translocations was shown by fluorescence in situ hybridization (FISH) in newborns born to mothers 31–40 years old vs. mothers 20–30 years old [25]. The data presented in The BioMadrid Study, focused on MN frequency that represented chromosomal breaks or losses, imply that the mother's age has no impact on the frequencies measured in newborns using conventional methods [26].

In this study we tested the hypothesis that the level of genetic damage, expressed as the frequency of micronuclei, varies in locations with different levels of air pollution. We have already studied the number of stable and unstable chromosomal aberrations using the FISH method in mothers and their newborns from Prague in the winter season of 2007–2008 [25]. One year later, we focused on a study of the micronuclei levels in these population groups in two different locations: in Prague – the capital city of the Czech Republic, surrounded by a densely populated area – and Ceske Budejovice – a regional city in the center of South Bohemia, surrounded by villages and mostly rural countryside. We evaluated the impact of B[a]P, PM2.5 and benzene, as well as the impact of vitamin intake, the BMI of the mothers before pregnancy, cotinine levels and the mothers' and fathers' ages on the micronuclei levels in newborns in a total of 178 mothers and their newborns. Our study is a part of a large molecular epidemiological project that concerns with the effect of air pollution also on other biomarkers including oxidative stress, PAH-DNA adducts, gene expression and genetic polymorphisms.

2. Materials and methods

2.1. Study subjects and sampling

The study was performed in the Czech Republic in collaboration with the University Hospital Motol in Prague and the Hospital Ceske Budejovice, a.s. The study populations recruited from both hospitals included 178 mothers and their newborns, all of Caucasian ethnicity. Mothers were aged 30.9 ± 4.1 years [median (range): 31.2 (18.3–49.0) years]. Newborns had a birth weight of 3435 ± 463 g [median (range): 3430 (2270–5150) g]. Information on the course and outcome of the pregnancy was obtained from medical records. Mothers who reported a viral infection during the last 3 months of pregnancy as well as mothers with low quality of the whole venous blood samples were excluded from the study along with their newborns. The main characteristics of the study groups from Prague and Ceske Budejovice were as follows:

Prague (the capital city in Central Bohemia with approximately 1,200,000 inhabitants): samples were collected between 6 November and 10 December 2008 (18 deliveries) and between 5 January and 24 March 2009 (68 deliveries). All 86 mothers lived in Prague during their pregnancy. Randomly chosen uncomplicated pregnancies (74% vaginal and 26% Caesarian births) were included in the Prague group.

Ceske Budejovice (a regional city in Southern Bohemia with approximately 100,000 inhabitants): samples were collected between 15 January and 31 March 2009 (92 deliveries). The group was split into mothers living in Ceske Budejovice (40 deliveries) and mothers living in villages and small towns (52 deliveries). All births in those groups were vaginal, from uncomplicated pregnancies.

Samples of maternal venous blood and umbilical cord blood were collected in sodium-heparinized tubes and stored at 4 °C until processing for the analysis of micronuclei. EDTA vacuettes were used to collect blood samples for the analysis of the levels of vitamin A and E. Plasma cotinine levels were measured to confirm the data on smoking obtained by questionnaires. Additional data obtained from the mothers' questionnaires and the newborns' health records were included into the analysis. These were: weight of the mothers before pregnancy for calculating their BMI, the age of the mothers and fathers, the education level of the mothers, weeks of gestation, type of delivery and birth weight of the newborns. All mothers involved in the study signed an informed consent form and could cancel their participation at any time during the study, according to the Helsinki II declaration. The ethical committee of the Institute of Experimental Medicine AS CR in Prague approved the study.

2.2. Monitoring of air pollution

Stationary monitoring of carcinogenic polycyclic aromatic hydrocarbons (c-PAHs, including benzo[a]pyrene, benz[a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[ghi]perylene, chrysene, dibenz[ah]-anthracene, and indeno[cd]pyrene), particulate matter of aerodynamic diameter <2.5 μm (PM2.5) and VOC to assess the level of ambient air pollution and the external exposure of the study subjects in both locations was performed in the center of Prague (Smichov) and Ceske Budejovice. The particulate air pollution was monitored by a versatile air pollution sampler (VAPS) [27]. The daily concentrations of c-PAHs, including B[a]P, were determined after c-PAH extraction from filters and quantitative chemical analysis performed by HPLC with fluorescence detection according to the EPA method [28]. VOC were detected at the same locations as VAPS monitoring by gas chromatography with flame/photo ionization detection. The levels of B[a]P and benzene, a representative c-PAH and VOC and PM2.5, for both locations in the years 2008 and 2009 were obtained from the Czech Hydrometeorological Institute. The mean concentrations of B[a]P, PM2.5 and benzene for a week, month and 3 months before delivery were calculated to estimate the individual exposure of each mother–newborn pair.

2.3. Processing of venous and cord blood

Whole venous blood and cord blood cultures were established within 12–24 h after blood collection, according to the method modified by Rossner et al. [29]. Two duplicate cultures were set up for each sample and cultivated at 37 °C. Thereafter, the cultures were incubated for 44 h, when cytochalasin B (Sigma, USA) was added to a final concentration of 5 μg/ml [30]. After 72 h of incubation, cultures were harvested by centrifugation, treated with a hypotonic solution of KCl (0.075 M) and fixed repeatedly with methanol/acetic acid. Finally, slides were prepared using the air-dry method.

2.4. Micronuclei automated image analysis

The analysis of MN slides, stained by DAPI [31], was performed using the automated scanning system Metafer 4, Version 3.2.1, from MetaSystems (Altlussheim, Germany) with a motorized Axio Imager Z1 microscope (Carl Zeiss, Germany) [32].

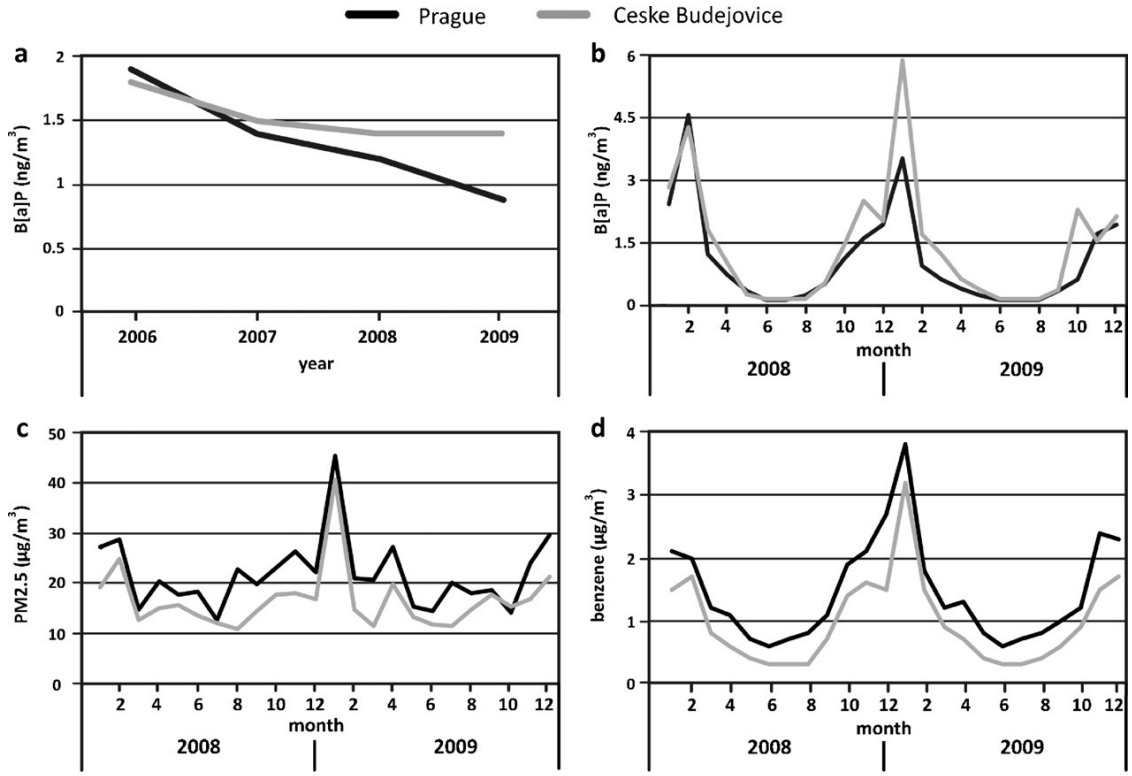

Fig. 1. Concentrations of the studied pollutants measured by stationary monitoring in Prague and Ceske Budejovice: (a) changes in the mean yearly concentrations of B[a]P during the years 2006–2009; mean monthly concentrations of B[a]P (b), PM2.5 (c) and benzene (d) during the years 2008 and 2009.

The classifier settings (MicroNuc Classifier, Version 3.5.104) for nuclei and micronuclei image processing operations were the same as in our previous studies [24]. These settings warrant QA/QC in choosing of objects. Scanning of binucleated cells (BNC) was performed at a final magnification of 100×. To eliminate false positives, all automated findings with one or more MN were checked and corrected if necessary. Whole galleries of images of BNC were stored for potential discussion in the future. Two thousands of BNC per person were analyzed in this study. The results were calculated as mean MN per 1000 BNC (MN/1000 BNC) and percentage of aberrant cells (%AB.C.).

2.5. Cotinine and vitamin assays

Cotinine as the major nicotine metabolite in venous and umbilical cord blood was analyzed by radioimmunoassay [33] to check the tobacco smoke exposure reported in the lifestyle questionnaires. We used the same cut-off value of cotinine in plasma (3 ng/ml) for significant exposure to tobacco smoke (smoking mothers) as in a previously published study focused on mothers and newborns [34].

Vitamins A and E in plasma were analyzed in a service laboratory of the Institute of Clinical Biochemistry and Laboratory Diagnostics of the General University Hospital in Prague by HPLC [35].

2.6. Statistical analysis

Statistical analyses were performed using SAS 9.1.3. software (SAS Institute, NC, USA). For the data that were not distributed normally, the nonparametric Mann–Whitney Sum *U*-test was used for the comparison between groups. Bivariate logistic regression was performed to assess the relationship between studied parameters (age of mothers, age of fathers, age of parents, cotinine level, BMI, gestation age and birth weight) and MN frequencies in the total group of mothers and newborns. For logistic regression two level scales (medians) were used; for cotinine, the cut-off value of 3 ng/ml for smoking mothers was used. Furthermore, we applied multivariate logistic regression analysis to assess the impact of selected factors (the studied pollutants, age of the mother, education and maternal cotinine) on the frequency of MN in mothers and newborns.

3. Results

The entire study was performed during the months in which the levels of air pollutants are elevated. Two hospitals were chosen for the collection of blood samples from newborns and their mothers. Prague was originally considered as a location with higher concentrations of pollutants and Ceske Budejovice as a regional city surrounded by villages with less pollution. In Fig. 1a the mean yearly concentrations of B[a]P measured by stationary monitoring in both locations during the years 2006–2009 are shown to demonstrate the trend of pollution. In both locations the situation was improving. This trend is more visible in Prague. As a result, Prague was less polluted by B[a]P in comparison with Ceske Budejovice (shown in detail in Fig. 1b). The opposite situation was observed for PM2.5 (Fig. 1c) and benzene (Fig. 1d).

The mean values of the exposure of mothers and their newborns to B[a]P, PM2.5 and benzene in the week, month and 3-month period before delivery are summarized in Table 1. This overview shows a significantly lower exposure to B[a]P in the Prague group in comparison with Ceske Budejovice, but the opposite situation for exposure to PM2.5 and benzene.

In Table 2A the basic characteristics of the mothers and their newborns from both locations are presented (number of subjects, mean, standard deviation (SD), median, maximum and minimum) for age, duration of gestation, BMI, cotinine and vitamin levels and birth weight. The data show significant differences in the levels of vitamins A and E in the mothers (higher in Prague). Furthermore, the data from questionnaires regarding the level of education show significantly higher education levels in Prague vs. Ceske Budejovice, $p < 0.05$ (data not shown). According to the questionnaires, 52 mothers from Ceske Budejovice were actually living in neighbor-

Table 1
Mean and median concentrations of B[a]P, PM2.5 and benzene a week, month and 3-month before delivery in Prague and Ceske Budejovice.

		Prague		Ceske Budejovice		*p*
		Mean ± SD	Median (range)	Mean ± SD	Median (range)	
B[a]P (ng/m^3)	Week	2.1 ± 1.7	1.2 (0.5–5.8)	2.1 ± 1.6	1.6 (0.7–9.2)	<0.05
	Month	2.1 ± 1.1	1.9 (0.8–3.8)	2.9 ± 1.8	1.7 (1.2–6.0)	<0.01
	Three-month	1.9 ± 0.5	2.2 (0.7–2.4)	3.2 ± 0.2	3.3 (3.0–3.6)	<0.001
PM2.5 (μg/m^3)	Week	31.1 ± 16.7	23.5 (15.5–71.5)	18.3 ± 10.3	15.5 (6.1–61.3)	<0.001
	Month	29.4 ± 8.2	25.0 (20.4–47.8)	23.4 ± 11.0	19.2 (11.1–42.4)	<0.001
	Three-month	27.0 ± 2.5	27.3 (22.1–31.7)	24.5 ± 0.7	24.7 (22.8–25.3)	<0.001
Benzene (μg/m^3)	Week	2.5 ± 1.5	2.1 (0.6–6.1)	1.6 ± 0.9	1.5 (0.5–4.9)	<0.001
	Month	2.7 ± 0.9	2.4 (1.2–4.2)	2.0 ± 0.8	1.7 (0.9–3.3)	<0.001
	Three-month	2.5 ± 0.5	2.8 (1.4–2.9)	2.1 ± 0.1	2.1 (1.9–2.1)	<0.001

hood villages, with possibly different air pollution (local heating). In Table 2B the same characteristics as in Table 2A are presented for subjects from Ceske Budejovice living in the city and in the surrounding villages. Differences in the median gestation weeks are evident in Table 2A, and this difference is significant when comparing Ceske Budejovice city vs. the villages. We can observe a decrease in the level of vitamin E in mothers between Prague, Ceske Budejovice-city and Ceske Budejovice-villages.

An overview of the mean values and SD of MN/1000 BNC and %AB.C. in the total group and subgroups from Prague and Ceske Budejovice, as well as the subgroups of mothers living in the city and in villages, is shown in Table 3. The data show significant differences between the groups from Prague and Ceske Budejovice in the mothers as well as their newborns. The comparison between the city and villages in Ceske Budejovice indicate that there are some differences, but they are not significant.

Table 4 shows MN frequencies per 1000 BNC and %AB.C. in the total studied group by characteristics of the study subjects. When divided by plasma cotinine levels, no difference in MN frequencies and %AB.C. were found in mothers, but significantly higher levels in MN frequencies were observed in newborns whose mothers were considered smokers by laboratory analysis ($p < 0.05$); but the difference in %AB.C. was on the borderline of significance ($p = 0.07$). An additional analysis of the impact of higher cotinine level on birth weight revealed that newborns of smoking mothers ($N = 21$, 6 from Prague and 15 from Ceske Budejovice) had a significantly lower birth weight by 7.4%, i.e. 257 g ($p < 0.05$) (data not shown). Comparing the age of the mothers showed significantly increased MN frequencies in mothers older than 30 years. Detailed separation into 5-year age brackets showed a continuous increase in genetic damage (data not shown). The age of the mothers had no impact on the MN frequencies in newborns. The frequencies of MN by BMI indicated the highest level of MN in mothers who were underweight before pregnancy and a decrease in the frequency of genetic damage with increasing BMI. This trend was not observed in newborns. Gestational age and the birth weight of the newborns did not

Table 2
Characteristics of the study subjects.

A

	Prague			Ceske Budejovice			*p*
	N	Mean ± SD	Median (range)	*N*	Mean ± SD	Median (range)	
Mothers							
Age (years)	86	31.2 ± 4.1	31.4 (21.0–49.0)	92	30.6 ± 4.15	30.8 (18.3–40.7)	0.39
Gestation (weeks)	86	39.2 ± 1.4	39.0 (35.0–42.0)	92	39.4 ± 1.1	40.0 (36.0–41.0)	0.26
BMI (before pregnancy)	86	22.8 ± 4.0	21.7 (15.8–35.3)	92	24.0 ± 5.6	22.5 (17.4–56.6)	0.14
Cotinine (ng/ml)	86	2.0 ± 11.3	0.2 (0.0–104.6)	92	8.3 ± 28.8	0.2 (0.1–150.3)	0.75
Vitamin A (mg/l)	85	0.6 ± 0.3	0.6 (0.1–1.9)	92	0.4 ± 0.2	0.4 (0.1–1.0)	<0.001
Vitamin E (mg/l)	85	15.6 ± 7.2	16.2 (2.0–38.9)	92	13.7 ± 6.3	13.6 (2.5–35.6)	<0.05
Newborns							
Birth weight (g)	86	3410 ± 494	3420 (2270–4720)	92	3457 ± 433	3430 (2580–5150)	0.59
Cotinine (ng/ml)	85	2.4 ± 14.2	0.3 (0.1–130.8)	92	8.1 ± 26.9	0.3 (0.1–146.8)	0.48
Vitamin A (mg/l)	83	0.3 ± 0.2	0.3 (0.0–0.8)	92	0.5 ± 0.3	0.5 (0.1–1.5)	<0.001
Vitamin E (mg/l)	83	3.9 ± 2.1	3.8 (0.2–9.9)	92	3.7 ± 1.6	3.6 (1.1–11.0)	0.59

B

	Ceske Budejovice-city			Ceske Budejovice-villages			*p*
	N	Mean ± SD	Median (range)	*N*	Mean ± SD	Median (range)	
Mothers							
Age (years)	40	30.1 ± 3.6	30.3 (18.3–37.8)	52	31.0 ± 4.5	31.6 (21.7–40.7)	0.33
Gestation (weeks)	40	39.7 ± 1.1	40.0 (37.0–41.0)	52	39.2 ± 1.1	39.0 (36.0–41.0)	<0.05
BMI (before pregnancy)	40	22.9 ± 3.5	22.1 (17.5–32.1)	52	24.8 ± 6.7	22.7 (17.4–56.6)	0.34
Cotinine (ng/ml)	40	5.2 ± 21.1	0.2 (0.1–131.4)	52	10.6 ± 33.6	0.2 (0.1–150.3)	0.32
Vitamin A (mg/l)	40	0.4 ± 0.2	0.3 (0.1–0.9)	52	0.4 ± 0.2	0.4 (0.1–1.0)	0.39
Vitamin E (mg/l)	40	13.9 ± 6.0	14.4 (2.5–35.4)	52	13.5 ± 6.5	12.9 (3.4–35.6)	0.35
Newborns							
Birth weight (g)	40	3480 ± 446	3430 (2750–4370)	52	3440 ± 427	3430(2580–5150)	0.71
Cotinine (ng/ml)	40	5.4 ± 20.5	0.3 (0.1–127.5)	52	10.2 ± 31.0	0.3 (0.1–146.8)	0.94
Vitamin A (mg/l)	40	0.5 ± 0.3	0.5 (0.2–1.3)	52	0.5 ± 0.3	0.4 (0.1–1.5)	0.64
Vitamin E (mg/l)	40	4.0 ± 1.6	3.9 (1.1–9.2)	52	3.5 ± 1.6	3.2 (1.4–11.0)	0.09

N: Number of subjects.

Table 3
MN frequencies per 1000 BNC and %AB.C. (mean ± SD) in the studied groups by location.

	Mothers			Newborns		
	N	MN/1000 BNC	%AB.C.	*N*	MN/1000 BNC	%AB.C.
Prague + Ceske Budejovice	178	7.38 ± 2.87	0.70 ± 0.26	178	3.02 ± 2.13	0.29 ± 0.20
Prague	86	8.35 ± 3.06[a]	0.80 ± 0.27[a]	86	2.17 ± 1.32[a]	0.21 ± 0.12[a]
Ceske Budejovice						
All	92	6.47 ± 2.35	0.61 ± 0.21	92	3.82 ± 2.43	0.37 ± 0.23
City	40	6.22 ± 2.08	0.59 ± 0.19	40	3.45 ± 1.92	0.32 ± 0.17
Villages	52	6.66 ± 2.53	0.62 ± 0.22	52	4.11 ± 2.75	0.40 ± 0.27

N: Number of subjects.
[a] Prague vs. Ceske Budejovice, $p < 0.001$.

Table 4
MN frequencies per 1000 BNC and %AB.C. (mean ± SD) in the total studied group by the characteristics of the study subjects.

Characteristics	Values	*N*	Mothers				Newborns			
			MN/1000 BNC	*p*	%AB.C.	*p*	MN/1000 BNC	*p*	%AB.C.	*p*
Cotinine (ng/ml)	≤3	157	7.35 ± 2.92		0.70 ± 0.26		2.95 ± 2.19		0.28 ± 0.21	
	>3	21[a]	7.62 ± 2.41	0.52	0.72 ± 0.24	0.67	3.52 ± 1.63	<0.05	0.33 ± 0.17	0.07
Age (years)	≤30	86	7.01 ± 2.93		0.67 ± 0.26		3.23 ± 2.50		0.31 ± 0.24	
	>30	92	7.72 ± 2.73	<0.05	0.74 ± 0.26	<0.05	2.83 ± 1.71	0.51	0.27 ± 0.16	0.73
BMI (before pregnancy)	<18.5	14	8.08 ± 4.24		0.75 ± 0.31		3.57 ± 3.60		0.35 ± 0.35	
	≥18.5–<25.0	118	7.45 ± 2.82	0.97	0.71 ± 0.26	0.87	2.88 ± 1.86	0.67	0.28 ± 0.18	0.65
	≥25.0–<30.0	31	7.06 ± 2.60	0.56	0.67 ± 0.25	0.52	3.22 ± 2.52	0.69	0.30 ± 0.24	0.76
	≥30.0	15	6.85 ± 2.29	0.57	0.65 ± 0.21	0.61	3.20 ± 1.60	0.28	0.31 ± 0.15	0.57
Gestational age (weeks)	≤37	17	7.31 ± 2.96		0.70 ± 0.27		2.53 ± 1.14		0.24 ± 0.11	
	>37	161	7.39 ± 2.87	0.85	0.70 ± 0.26	0.79	3.07 ± 2.21	0.60	0.29 ± 0.21	0.41
Birth weight (g)	<3000	34	7.76 ± 3.10		0.72 ± 0.25		2.80 ± 1.46		0.26 ± 0.14	
	≥3000–<4000	126	7.35 ± 2.87	0.26	0.70 ± 0.27	0.52	3.08 ± 2.34	0.98	0.30 ± 0.22	0.88
	≥4000	18	6.86 ± 2.38	0.45	0.67 ± 0.22	0.39	3.00 ± 1.70	0.69	0.28 ± 0.15	0.95
Delivery mode[b]	Vaginal	64	8.41 ± 3.11		0.80 ± 0.27		2.18 ± 2.20		0.21 ± 0.13	
	Caesarian	22	8.20 ± 2.98	0.70	0.79 ± 0.28	0.66	2.14 ± 1.32	0.93	0.20 ± 0.12	0.96

N: Number of subjects.
[a] Birth weight of children was significantly lower ($p < 0.05$) in comparison with the group of nonsmoking mothers.
[b] Only for the group from Prague.

Table 5
Bivariate logistic regression of the studied parameters on the frequency of MN.

	Mothers		Newborns	
	OR[a] (95% CI)[b]	*p*	OR[a] (95% CI)[b]	*p*
Age – mothers	2.00 (1.10–3.66)	<0.05	0.73 (0.40–1.32)	0.29
Age – fathers	N/A	N/A	1.21 (0.66–2.22)	0.55
Cotinine	1.02 (0.41–2.56)	0.97	3.89 (1.25–12.07)	<0.05
BMI	0.96 (0.53–1.73)	0.88	1.15 (0.63–2.07)	0.65
Gestational age	1.42 (0.70–2.88)	0.33	1.74 (0.86–3.53)	0.13
Birth weight	0.76 (0.42–1.37)	0.35	1.10 (0.60–1.97)	0.78

N/A: Not applicable.
[a] Odds ratio.
[b] Confidential interval.

show associations with the frequency of MN; however, our study group included only one case of low birth weight (LBW). The impact of delivery mode in Table 4 is shown for the Prague group only, because in Ceske Budejovice all deliveries were vaginal. Comparing the frequency of MN in mothers and newborns in 64 vaginal birth cases vs. 22 Caesarian births did not show any impact of delivery type.

Table 5 summarizes the impact of the studied factors on the frequency of MN in both groups by bivariate logistic regression. The results are generally in agreement with the data reported in the previous tables and confirm the impact of the mothers' ages on their MN levels and the impact of smoking on the MN levels of newborns. An impact of BMI on MN frequency in the mothers was not confirmed. Table 6 shows the impact of the selected parameters on the frequency of MN in mothers and newborns by multivariate logistic regression. These data indicate a significant impact of benzene measured 2 months before delivery in the group of moth-

Table 6
Multivariate logistic regression of the studied parameters on the frequency of MN.

	OR[a] (95% CI)[b]	*p*
Mothers		
Benzene[c]	2.11 (1.14–3.91)	<0.05
Age	1.80 (0.95–3.39)	0.07
Education (higher)	0.59 (0.29–1.21)	0.15
Cotinine	1.02 (0.39–2.72)	0.96
Newborns		
B[a]P[d]	3.07 (1.63–5.77)	<0.001
Age	0.78 (0.41–1.50)	0.45
Education (higher)	1.06 (0.52–2.18)	0.87
Cotinine	3.14 (0.96–10.31)	0.06

[a] Odds ratio.
[b] Confidential interval.
[c] Two months before delivery.
[d] Three months mean before delivery.

ers ($p < 0.05$) and a significant impact of the 3 months mean B[a]P before delivery in the group of newborns ($p < 0.001$). The impact of the mothers' ages on their MN levels and the impact of the mothers' smoking on the MN levels of their newborns are on the borderline of significance. The effect of birth weight on MN frequency was not significant (data not shown).

4. Discussion

In the present study we investigated an association between the MN frequency in mothers and their newborns and different types of air pollution in two locations in the Czech Republic. The study locations, Prague and Ceske Budejovice, displayed opposite trends in the levels of the studied pollutants. Significantly higher mean exposure to B[a]P was measured in the study group from Ceske Budejovice a week, month and 3 months before delivery; nevertheless, the Prague group was significantly more exposed to PM2.5 and benzene. The measured levels of MN in newborns were in agreement with our expectations for the impact of different levels of B[a]P on chromosomal aberrations in humans [21,24,36]. The results of comparing MN frequency in Ceske Budejovice city and in the neighboring villages probably reflect differences in the heating systems in the villages (use of low quality brown coal and wood instead of natural gas or central heating). Other potential explanation may be related to agricultural exposures, such as pesticides [37]. The genetic damage measured in the mothers showed an opposite trend in comparison with that of newborns. Similar results were reported in a study by Pedersen et al. [10], in which significantly increased MN frequencies were found among newborns whose mothers reported high traffic near their homes, whereas maternal MN showed a non-significant but opposite trend.

The different type of air pollution in studied regions and different sensitivity of the study groups are main explanations of our results. Our previous study by FISH and oxidative damage in a group of city policemen indicated the impact of higher exposure to PM2.5 on the genomic frequency of translocations ($F_G/100$) and oxidative damage to DNA [38]. As mentioned in the introduction, conclusions from European studies on long-term exposure to PM indicate a significant health risk [4]. Concerning benzene, most of the published studies have focused on occupationally exposed workers, and the exposure to benzene is reported as being several parts per million per m^3. Studies concerning exposure to low levels of benzene in concentrations of several parts per billion per m^3 (1 ppb = 3.2 $\mu g/m^3$) in association with the micronucleus test are relatively scarce and report conflicting results [39,40]. It is known that benzene crosses the human placenta and impacts the formation of chromosomal aberrations in peripheral lymphocytes of individuals exposed to high levels of benzene. The results are not so clear with lower levels of benzene exposure as environmental factors and exposure to other agents may interact with benzene [5]. We can speculate that the protective capacity of the placenta was adequate to lower the exposure of newborns to benzene and PM2.5 in our study.

In general, when comparing mothers from Prague and Ceske Budejovice, it is important to mention that in Prague (12× more populated than Ceske Budejovice) people are generally closer to the sources of pollution, mainly caused by traffic. However, the Prague mothers in our study had a significantly higher level of education and probably a higher living standard, which we can infer by their significantly higher intake of vitamins or the lower number of smoking mothers. This may have a positive effect on their newborns.

Body mass index was another factor we studied. The commentary by Battershill et al. summarizes the association between BMI and genotoxicity biomarkers, with variable results [41]. In our study the highest frequency of MN was measured for mothers underweight before pregnancy. A similar result was recently presented by Lope et al. [26]. An inverse association between weight loss and DNA damage (urinary level of 8-hydroxydeoxyguanosine) was shown in a study by Mizoue et al. [42]. Bivariate logistic regression did not confirm this negative association in our study, but additional analysis with equal and higher numbers of samples in each weight category is needed.

Current knowledge about the impact of smoking on MN frequency is briefly summarized in the introduction. In our study no difference in MN frequencies was found in mothers by plasma cotinine levels, which is in agreement with the generally accepted data [20]. In previously published study, the number of MN was significantly higher in the 41 cord blood samples from mothers who smoked during pregnancy [22]. Our data obtained by comparison of MN/1000 BNC and, %AB.C. ($p < 0.05$, and $p = 0.07$, respectively) indicate possible higher genetic damage in newborns of smoking mothers. The significant reduction in the mean birth weight of infants of actively smoking mothers observed by Dejmek et al. [43], as well as recently by Anderka et al. [44], is in agreement with our data, which showed the birth weight of this group to be lower by 7.4% ($p < 0.05$) in comparison with the newborns of nonsmoking mothers.

Age is one of the most important factors that could affect micronuclei data. Lower MN frequency in the total group of newborns in this study is in agreement with previous published data obtained by automated image analysis and expends the set of 445 subjects (age 6–65 years) [24]. Our results obtained from mothers are in agreement with published data from visual scoring as well as with the significant differences observed in the frequency of micronuclei between mothers and newborns [10,23]. The analysis of the impact of the age of the mothers and fathers in our study showed no impact of the age of any group on the MN frequency in newborns. We have already reported a significant increase in the levels of stable chromosomal aberrations, represented by translocations analyzed by the FISH method, in newborns born to mothers 31–40 years old vs. mothers 20–30 years old [25]. The data obtained for acentric fragments, which represent unstable chromosomal aberrations, obtained in this study are in agreement with our observations for micronuclei in the present study as well as with data from the BioMadrid Study [26].

In conclusion, our study presents the results of automated image analysis of MN from 356 individuals (mothers + newborns) sampled in two regions of the Czech Republic with different types of air pollution. The Prague group was affected by complex exposure with higher concentrations of PM2.5 and benzene and the group from Ceske Budejovice by a higher level of B[a]P. Our study indicates the differing sensitivity of the study groups to various mixtures of carcinogenic pollutants and suggests a significant impact of lifestyle factors. Studying the impact of the mothers' smoking suggests possible higher genetic damage in their newborns, and a significant impact of smoking on birth weight. Our analyses demonstrate no connection between the mothers' and fathers' ages on the frequency of MN in newborns. For future study, we propose a comparison with a region with an extremely high concentration of air pollution and detailed analysis of the dietary factors.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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